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THE EFFECT OF MIDBRAIN TRANSECTION ON ACTH RELEASE

L. MARTINI, A. PECILE, S. SAITO[1] AND F. TANI

Department of Pharmacology, University of Milan, Milan, Italy

ABSTRACT

The influence of mesencephalic structures on the hypothalamus-anterior pituitary-adrenal cortex system was investigated in rats subjected to midbrain section by observing the adrenal ascorbic acid response to the stresses of ether anesthesia, unilateral adrenalectomy and abdominal surgery. There was no response indicative of increased secretion of ACTH whereas intact rats, sham-operated rats, and rats with the spinal cord sectioned at C_7 responded with a marked fall in adrenal ascorbic acid. The defect in the rats with midbrain section was not at the adrenal cortical level, since the response to injected ACTH was normal, nor did the anterior pituitary appear to be insensitive, since there was a significant although diminished response to administered Pitressin. It was concluded that the midbrain lesion had interfered with some neural mechanism which has the capacity of activating the hypothalamus-anterior pituitary system.

A CONSIDERABLE body of evidence has accumulated to indicate that the hypothalamus exerts an important influence on the secretion of adrenocorticotropic hormone (ACTH) by the release of an activating neurohumoral substance into the hypophysial portal vessels which pass from the *tuber cinereum* to the anterior lobe (1, 2). This represents a "final common path" (3) for the great variety of stimuli which induce the secretion of ACTH. Work has been initiated in a number of laboratories to elucidate the role of stimuli reaching the hypothalamus from lower or higher centers of the nervous system and terminating on the "final common path," but the results thus far have been rather confusing.

Heat (4) on stimulation of the septum obtained a drop in blood eosinophils suggesting the release of ACTH; conversely Moll (5) found that septal lesions have an inhibitory effect on adrenal compensatory hyper-

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[1] Permanent address: Department of Surgery, Gumna University Medical School, Maebashi, Japan.

trophy;[2] Pool *et al.* (7), Endröczi *et al.* (8, 9) and Ganong *et al.* (10) showed that stimulation of the orbitofrontal parts of the brain activates the pituitary-adrenal system and enhance the ACTH releasing activity of adrenaline. Mason (11, 12, 13) reported that in monkeys the stimulation of the hippocampus and of the preoptic region lowers plasma 17-hydrocorticosteroid levels, whereas the stimulation of other lymbic areas (amygdaloid complex) results in significant plasma hydroxycorticosteroid elevation. Amygdala lesions are followed in cats by extreme increase in corticoid secretion as shown by Endröczi (14). Fornix section disrupts the diurnal rhythm of urinary 17-hydrocorticosteroid output (11, 12, 13) and according to Egdahl *et al.* (15) decortication, decortication plus removal of basal ganglia, hippocampus and fornix, and ablation of the thalamus do not result in diminution of the adrenal response to operative trauma in dogs.

The brain stem reticular formation exerts a critical influence in the motor and sensory spheres, but little is known concerning reticulo-hypothalamic relationships (5, 16, 17, 18). The purpose of this study was to investigate the influence of the reticular system and other mesencephalic structures on the hypothalamus pituitary-adrenal system of the rat.

METHODS

These experiments were carried out in male rats of the Sprague-Dawley strain weighing 150–250 gm. The operative procedure was as follows: rats were anesthetized with ether, a sagittal incision was made in the scalp and the muscles were removed from the occipital bone, a hole was then made in the bone by means of a little drill, the hole was enlarged to about 0.8–1 cm. in diameter, the dura was then opened and the supratentorial part of the midbrain exposed. Midbrain incision was made at the tentorial level with a little scalpel; the level and the plane of transection varied somewhat in different animals but the transection was always complete (Fig. 1). After midbrain transection rats resemble a "reticular" preparation as described by Lindsley (19). They were kept in single cages at 25° C and given Pencillin subcutaneously once daily. They were fed by stomach tube with milk plus aminoacids, glucose, salts and vitamins. The mortality rate after midbrain transection was rather high (50%), most of the animals dying within the first 3 days. Experiments in the operated animals were usually performed between the 5th and the 25th day after midbrain transection because it has been shown that comparable results were obtained in this period of time.

A potential complication in the study of the effects of midbrain transection on ACTH release in the rat is related to the position of the pituitary which is located under the midbrain region. In order to eliminate the possibility of a blockade of ACTH release due to pituitary damage, the brains were removed at autopsy, and the glands were examined with a magnifying glass. Animals with damaged pituitaries (10% of surviving animals) were discarded.

Control experiments were performed: 1) in sham-operated animals in which the operative procedure was interrupted after having exposed the midbrain; 2) in spinal rats in which the spinal cord had been transected at the C_7 level.

[2] It is worth mentioning that the stimulation of the septal region is followed in Rhesus monkeys by an antidiuretic response mediated through the release of the antidiuretic hormone (6), the postulated neurohumor which activates the release of ACTH (1, 2)

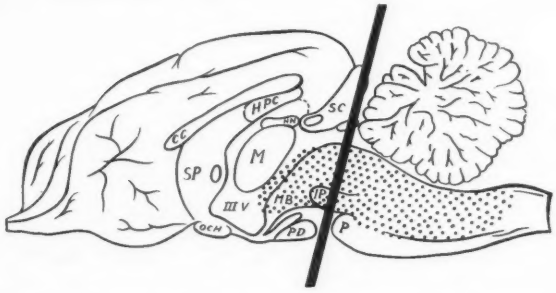

FIG. 1. Mesencephalic region in the rat. Abbreviations: CC, Corpus Callosum; SP, Septum pellucidum; OCh, Optic chiasma; III V, Third ventricle; M, Massa intermedia; HPC, Hippocampus; HN, Nucleus habenulae; SC, Superior colliculus; MB, Mammillary bodies; PD, Pars distalis; IP, Nucleus interpeduncularis; P, Pons. The stippled area indicates the localization of the reticular formation of the brain stem.

To test the reactivity of the hypothalamic-pituitary adrenal system of the midbrain sectioned animals, the following stresses were applied: 1) Unilateral adrenalectomy under ether anesthesia. 2) Laparatomy and exposure of the intestines to the drying effect of a lamp for 5 minutes. 3) Pitressin[3] injection (0.3 U/rat intravenously). The sensitivity of the midbrain sectioned rat to ACTH (2 and 4 mU/rat intraperitoneally) was also studied.

The ACTH releasing effect of the different procedures was evaluated by means of adrenal ascorbic acid depletion method (20); right adrenalectomy was performed under ether anesthesia to obtain a control level of ascorbic acid; the animals were killed by decapitation 2 hours after the different treatments and the left adrenal was immediately removed. The adrenal glands were rapidly dissected free of fat and weighted on a torsion balance. Their content of ascorbic acid was then estimated by the method of Roe and Kuether (21).

Liver glycogen determinations were carried out according to the method of Stafford and co-workers (22).

The significance of the differences between the means obtained was calculated by Student's "t" test.

RESULTS

The surgical procedure of transecting the midbrain under ether anesthesia was associated with a stress reaction, as shown by a marked drop of adrenal ascorbic acid and a significant decrease in liver glycogen concentration immediately after the transection. Liver glycogen returned slowly to normal in the following days, reaching its original value on the 5th day (Fig. 2). For this reason experiments in the sectioned animals were usually performed starting from the 5th postoperative day.

In normal, sham-operated and spinal rats a definite and significant fall of adrenal ascorbic acid followed the stress of ether anesthesia and unilateral adrenalectomy (Table 1). In the midbrain animals the same stimuli did not induce any adrenal ascorbic acid depletion.

[3] Pitressin (a commercial antidiuretic hormone preparation) Lot #31846 was kindly supplied by Dr. D. A. McGinty of Parke, Davis and Co.

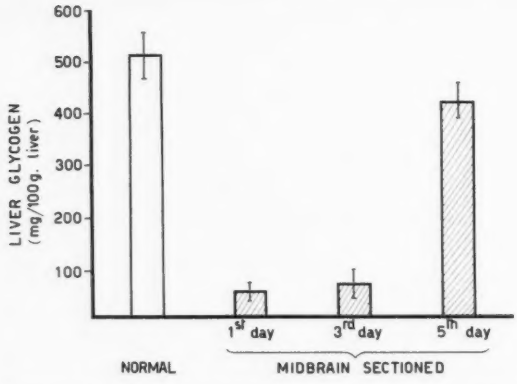

FIG. 2. Liver glycogen in normal and midbrain sectioned rats. (Each point represents the mean of 8 animals. The vertical lines are standard errors.)

Table 2 summarizes the results obtained in the laparatomy experiments. In normal rats the stress of ether anesthesia and unilateral adrenalectomy plus laparatomy is followed by a highly significant drop of adrenal ascorbic acid concentration ($P < 0.001$).

In the midbrain sectioned group laparatomy (plus unilateral adrenalectomy) was carried out without ether anesthesia and the intestines were exposed to the drying effect of the operating lamp for 5 minutes, but no drop of adrenal ascorbic acid occurred following this procedure.

Figure 3 shows the sensitivity to exogenous ACTH of midbrain sectioned rats. It is evident that the adrenals of the decerebrate animals still show an ascorbic acid fall following the administration of the hormone. These results seem to indicate that the block of ACTH secretion observed in midbrain sectioned rats after unilateral adrenalectomy and laparatomy is not related to a block of ACTH activity at the adrenal level but that it is probably due to an impairment of a midbrain-hypothalamic mechanism.

Table 3 shows the results obtained when Pitressin was injected intravenously into normal and midbrain sectioned rats after ether anesthesia and unilateral adrenalectomy. This stimulus was chosen because it seems

TABLE 1. ADRENAL ASCORBIC ACID DEPLETION PRODUCED BY ETHER ANESTHESIA AND UNILATERAL ADRENALECTOMY IN NORMAL, SHAM-OPERATED, SPINAL AND MIDBRAIN SECTIONED RATS

	No. of rats	Adrenal ascorbic acid concentration (mg./100 gm. adrenal)		P
		1st adrenal	2nd adrenal	
Normal	14	416 ± 10 (*)	314 ± 14	<0.001
Sham-operated	8	420 ± 22	332 ± 20	<0.02
Spinal	14	383 ± 15	286 ± 12	<0.01
Midbrain	14	396 ± 22	426 ± 8	

(*) Mean $\pm$ S.E.

TABLE 2. ADRENAL ASCORBIC ACID DEPLETION PRODUCED BY UNILATERAL ADRENALECTOMY PLUS LAPARATOMY IN NORMAL AND MIDBRAIN SECTIONED RATS

	No. of rats	Adrenal ascorbic acid concentration (mg./100 gm. adrenal)		P
		1st adrenal	2nd adrenal	
Normal	13	411 ± 24 (*)	280 ± 27	<0.001
Midbrain	14	398 ± 21	394 ± 20	

(*) Mean ± S.E.

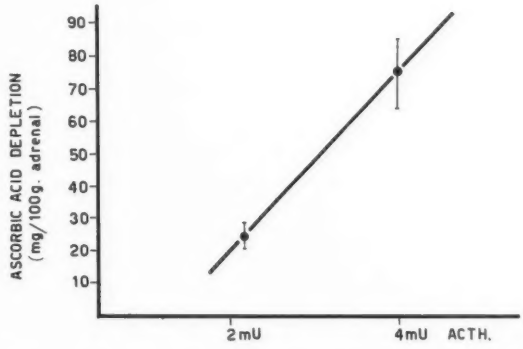

FIG. 3. ACTH sensitivity of midbrain sectioned rats (Each point represents the mean of 8 animals. The vertical lines are standard errors).

TABLE 3. ADRENAL ASCORBIC ACID DEPLETION PRODUCED BY ETHER ANESTHESIA, UNILATERAL ADRENALECTOMY PLUS PITRESSIN (0.3 U.I.V.) IN NORMAL AND MIDBRAIN SECTIONED RATS

	No. of rats	Adrenal ascorbic acid concentration (mg./100 gm. adrenal)		P
		1st adrenal	2nd adrenal	
Normal	12	411 ± 8 (*)	302 ± 14	<0.001
Midbrain	19	391 ± 9	340 ± 12	<0.02

(*) Mean ± S.E.

to act directly at the hypophysial level since previous investigations have shown that Pitressin releases ACTH in hypophysectomized rats bearing a functional pituitary graft in the anterior chamber of the eye (2, 23), in hypothalamic lesioned animals (24), in morphine-blocked guinea pigs (25) and in "in vitro" cultures of pituitary tissue (26). In the midbrain sectioned rats, as well as in the normal animals, Pitressin administration was followed by a significant fall in adrenal ascorbic acid concentration. The adrenal response was somewhat diminished in the sectioned animals as shown by the different degree of significance of the results obtained.

DISCUSSION

The experiments here presented have shown that midbrain sectioned rats are unable to release ACTH after the exposure to severe stress such as unilateral adrenalectomy and abdominal surgery. The defect in these rats is not at the adrenal level because the adrenal gland still responds to exogenous ACTH with a fall in ascorbic acid concentration; nor is the sensitivity of hypophysial cells greatly diminished after midbrain transection as shown by the response of the operated animals to Pitressin administration. These results are in agreement with the previous reports of Anderson and co-workers (16, 17) who have shown in midbrain sectioned dogs that severe stresses do not produce any modification in urinary steroids and of Sayers (27, 28) who has found that section of the brain stem at the upper level of the pons abolishes the stimulatory action of ether anesthesia on the pituitary. In agreement with these results Newmann and co-workers (18) have found that midbrain and supramammillary diencephalic lesions significantly reduce hydrocortisone secretion in the cat and Moll (5) observed that electrolytic mesencephalic lesions block the compensatory hypertrophy following unilateral adrenalectomy.

The ability of Pitressin to release ACTH in midbrain sectioned animals is in conflict with a previous observation (27). In the experiments here presented Pitressin was however less active in midbrain sectioned than in normal animals. This result as well as the observation that the rise in plasma hydrocortisone concentration resulting from intravenous injection of Pitressin in man is significantly suppressed by morphine (29), a drug which has an inhibiting action on mesencephalic structures, seem to indicate that the pituitary is less sensitive to its physiological activator when mesencephalic structures are lesioned or inhibited.

On evidence derived from pituitary transplantation and stalk section experiments Fortier (30) and Fortier *et al.* (31) have divided stresses into two groups: 1) "neurotropic stresses," those that elicit ACTH discharge by an action through the central nervous system and 2) "systemic stresses" (such as laparatomy and unilateral adrenalectomy), which may act by releasing chemical substances into the circulation or by producing metabolic changes. The experiments here presented seem to indicate that also "systemic" or "metabolic" stresses act through the excitations of nervous pathways which ultimately lead to the release of the anterior pituitary stimulating substance(s) from the median eminence (1, 2). Guillemin *et al.* (32) have advanced the hypothesis that under certain circumstances nonspecific stimuli may activate the secretion of ACTH through the release of hypophysiotropic material of extrahypothalamic origin. The present study did not corroborate this suggestion, since it was observed that laparatomy and unilateral adrenalectomy, which are severe "systemic stresses" in normal rats, were ineffective in stimulating ACTH secretion after midbrain transection.

REFERENCES

1. Martini, L.: in S. B. Curri, and L. Martini: Pathophysiologia Diencephalica, Springer, Wien, 1958, p. 229.
2. Casentini, S., A. De Poli, S. Hukovic and L. Martini: *Endocrinology* **64**: 483. 1959.
3. Sayers, G., E. S. Redgate and P. C. Royce: *Ann. Rev. Physiol.* **20**: 243. 1958.
4. Heat, R. G.: Studies in schizophrenia. A multidisciplinary approach to midbrain relationships. Commonwealth Found., Cambridge (U.S.A.), 1954.
5. Moll, J.: *Z. Zellforsch.* **49**: 515. 1959.
6. Dingman, J. F., E. Gaitan, A. Arimura and R. G. Heat: Endocrine Society 41st Meeting, Abstracts of papers, 1959, p. 51.
7. Pool, J. L., W. K. Clark, P. Hudson and M. Lombardo: in Hypothalamic-hypophysial interrelationships, Charles C Thomas Publisher, Springfield, Illinois, USA, 1956, p. 114.
8. Endrõczi, E. and K. Lissak: *Endokrinologie* **30**: 310. 1953.
9. Endrõczi, E., S. Kovács and B. Bohns: *Acta Physiol. Hung.* **14**: 39. 1958.
10. Ganong, W. F. and A. Goldfien: Endocrine Society 41st Meeting, Abstracts of papers, 1959, p. 29.
11. Mason, J. W.: *J. Clin. Endocrinol. and Metab.* **16**: 914. 1956.
12. Mason, J. W.: *Endocrinology* **63**: 403. 1958.
13. Mason, J. W., W. J. H. Nauta, J. V. Brady and J. A. Robinson: Endocrine Society 41st Meeting, Abstracts of papers, 1959, p. 29.
14. Endrõczi, E., K. Lissak, A. Tigyi and C. Szep: *Acta physiol. Hung.* **6**: 19. 1954.
15. Egdahl, R. H., J. L. Story and J. C. Melby: *Fed. Proc.* **17**: 435. 1958.
16. Anderson, E., K. Knowlton, W. T. Spence, S. M. McCann, G. L. Laqueur, D. Mc K. Rioch and W. Haymaker: *Acta Neuroveg.* **12**: 53. 1955.
17. Anderson, E., R. W. Bates, E. Hawthorne, W. Haymaker, K. Knowlton, D. Mc K. Rioch, W. T. Spence and H. Wilson: *Recent Progr. Hormone Res.* **13**: 21. 1957.
18. Newmann, A. E., E. S. Redgate and G. G. Farrell: *Endocrinology* **63**: 723. 1958.
19. Lindsley, D. B., L. H. Schreiner, W. B. Knowles and H. W. Magoun: *Electroencephalog. & Clin. Neurophysiol.* **2**: 483. 1950.
20. Sayers, M. A., G. Sayers and L. A. Woodbury: *Endocrinology* **42**: 379. 1948.
21. Roe, J. H. and C. A. Kuether: *J. Biol. Chem.* **147**: 399. 1943.
22. Stafford, R. O., L. E. Barnes, B. J. Bowman and M. M. Meinzinger: *Proc. Soc. Exp. Biol. and Med.* **89**: 371. 1955.
23. Martini, L. and A. De Poli: *J. Endocrinol.* **13**: 229. 1956.
24. McCann, S. M.: *Endocrinology* **60**: 664. 1957.
25. Sobel, H., S. Schapiro and J. Marmorston: *Am. J. Physiol.* **195**: 147. 1958.
26. Saffran, M., A. V. Schally and B. G. Benfey: *Endocrinology* **57**: 439. 1955.
27. Sayers, G.: *Ciba Foundation Colloquia on Endocrinology* **11**: 138. 1957.
28. Royce, P. C. and G. Sayers: *Endocrinology* **63**: 794. 1958.
29. McDonald, R. K., F. T. Evans, V. K. Weise and R. W. Patrick: *Fed. Proc.* **17**: 109. 1958.
30. Fortier, C.: *Endocrinology* **49**: 782. 1951.
31. Fortier, C., G. W. Harris and I. R. McDonald: *J. Physiol.* **135**: 344. 1957.
32. Guillemin, R., W. R. Hearn, W. R. Cheek and D. E. Housholder: *Endocrinology* **60**: 488. 1957.

SOME EFFECTS OF ADRENALECTOMY AND PREDNISOLONE ADMINISTRATION ON EXTRACELLULAR FLUID AND BONE COMPOSITION IN THE RAT[1]

ANDRÉ B. BORLE,[2] GEORGE NICHOLS, JR.[3] AND MORRIS J. KARNOVSKY

Departments of Biological Chemistry, Medicine, and Pathology, Harvard Medical School, Boston, Massachusetts

ABSTRACT

Plasma and bone electrolyte concentrations were measured in adrenalectomized and Prednisolone treated male rats in order to investigate what influence the absence of adrenal secretion and excess glucocorticoid might have on the distribution of ions between the extracellular fluid and the bone mineral. Three groups of rats were studied for 7 days. One group served as controls, the second was adrenalectomized, and the third received 10 mg of Prednisolone daily.

In the adrenalectomized group, hyponatremia was reflected by a decreased sodium concentration in the bone mineral. In the steroid treated animals, despite a fall in plasma calcium, the calcium concentration in the bone mineral increased significantly, while the sodium concentration did not change in either plasma or bone. From these data it would appear that the ionic shifts between bone mineral and extracellular fluid are not solely the result of physicochemical equilibrium. Rather, they appear to be at least in part controlled by local factors in the organic phase of bone which in turn may be modified by an excess of a circulating glucocorticoid such as prednisolone.

FOR many years, bone has been known to play an important role in electrolyte metabolism. In 1933, Hastings and Huggins (1) showed that the large store of calcium in bone could be rapidly mobilized to support the extracellular fluid calcium concentration. More recently, work from this laboratory and others has indicated that the store of sodium in bone could be decreased in sodium depletion and increased in sodium loading (2, 3, 4). In both instances it has been postulated that these exchanges of ions were governed by the physicochemical laws of equilibrium between a crystal and its surrounding fluid—a point of view supported by *in vitro* studies of exchanges of calcium, sodium, phosphate and citrate with synthetic hydroxyapatite (5, 6, 7, 8).

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[2] Present address: Clinique Therapeutique, Universitaire Hopital Cantonal, Geneve, Switzerland.

[3] Markle Scholar in Medical Science.

In addition to these changes, adrenalectomy has been shown to decrease radio-sodium uptake by bone in the dog (9) and to result in decreased bone sodium concentration in the rat (10). On the other hand, the effects of adrenal steroid administration on bone mineral composition have not been extensively studied although Clark and Geoffroy (11) have presented evidence indicating a decreased skeletal uptake of Ca^{45} and an increased urinary excretion of the isotope in rats treated with glucocorticoids. Their results were especially striking when prednisolone was the steroid administered.

The present study was undertaken for two reasons: to obtain more information concerning the influence of adrenal activity on the distribution of ions between the extracellular fluid and bone mineral; and to see whether the changes that have been observed were a result of a direct effect upon the skeletal tissue or secondary to changes in electrolyte metabolism of the body as a whole.

EXPERIMENTAL PROCEDURES

Thirty 130 day old albino male rats from Rockland Farm, N. Y., weighing about 300 gm. each, were divided into three groups. The first served as controls, the second was adrenalectomized on the first day of the experiment, and the third received 10 mg. of prednisolone (Meticortelone Schering) subcutaneously every day for the experimental period of seven days. This steroid was selected because, according to Clark's findings (11), it was the most effective in reducing the bone radiocalcium uptake and has been reported to have little effect on serum sodium and potassium (12). All animals were fed Purina lab. chow *ad lib.* For the first two days, they received 0.4% NaCl solution to drink, then tap water until sacrifice. For the last 24 hours, they were fasted in metabolic cages and urine was collected in order to measure electrolyte balance. On the seventh day, the animals were anesthetized with Nembutal and exsanguinated through the abdominal aorta. Both femurs of each animal were dissected free, the epiphyses and metaphyses were clipped off, the bone marrow and periosteum removed, and the shafts placed in tared stoppered weighing bottles.

An additional experiment was done on 7 male rats of the same strain weighing 100 gm. each in order to examine the effects of prednisolone on the histology and composition of bone from young growing animals. Four of these rats were given prednisolone for 5 days before sacrifice: 10 mg. on the first day and 5 mg. on each succeeding day. Three served as controls and were pair-fed Purina lab. chow to the steroid treated animals throughout the experimental period. All were given tap water *ad lib.* In these experiments the animals had access to food until the time of sacrifice. The rest of the experimental procedure was the same as for the 300 gm. rats.

METHODS AND CALCULATIONS

The methods for plasma and bone electrolytes and the calculations used to determine the size and the composition of the extracellular compartment have already been described (2, 3, 13, 14). Whole blood pH was measured anaerobically. The Donnan factors used were taken from Manery (15), and the ionized fraction of calcium in serum was calculated from the nomogram of McLean and Hastings (16). Histological sections of bone from young and adult rats from controls and the steroid treated group were made by standard methods.

RESULTS

a) *Condition of the animals:* At the end of the experiment, the control group showed a mean weight loss of 4 gm. The adrenalectomized group had lost an average of 49 gm. and showed signs of acute adrenal insufficiency. The third group which received 10 mg. of prednisolone daily, lost 75 gm., almost twice as much as the adrenalectomized animals.

b) *Plasma:* The means and standard deviations of the plasma values for each group are presented in Table 1. The adrenalectomized rats had a blood pH of 7.31, which was substantially lower than in the two other groups (7.38). When compared with controls, they showed a significant hyponatremia (−7.3%), hyperkalemia (+58.8%), and hypochloremia (−8.1%) as would be expected in adrenal insufficiency. Although their

TABLE 1. PLASMA COMPOSITION

	pH	H_2O Gm/L	Na m.eq/L	K m.eq/L	Total Ca m.eq/L	Cl m.eq/L	P mM/L
Control							
Mean (10)	7.38	952	145.4	3.9	5.37	101.7	2.25
SD	.05	2.5	2.9	.38	.62	3.3	.23
Adrenalectomy							
Mean (10)	7.31	945	134.6	6.0	5.19	93.5	3.06
SD	.04	3.6	2.4	3.7	.17	2.7	.20
Prednisolone							
Mean (10)	7.38	941	145.3	3.7	4.86	98.5	1.97
SD	.04	4.1	3.2	.49	.26	4.4	.26

total plasma calcium concentration did not change, the total plasma phosphorus increased 36%. This increase may be partially explained by impaired renal function and tissue breakdown.

In the steroid treated group, plasma water was low, 941 gm./L compared with 952 gm./L in the controls, while the sodium and potassium did not drop significantly. The total calcium, however, decreased by 9.5% and the effect on the calculated ionized fraction was still more striking, −12.5%. In an attempt to determine whether the decrease in plasma calcium concentration observed was due to decreased intestinal absorption, decreased bone mobilization or increased excretion of calcium, urines were collected for the last 24 hours before sacrifice. Unfortunately, due to the small output of these animals and the high concentration of phosphate in the urine, which interferes with calcium measurement, the results were inconclusive and have therefore been omitted. However, Clark *et al.* (17) did not find any rise in the total output of calcium in their prednisolone treated rats, and therefore, this possibility seems unlikely.

c) *Extracellular fluid:* Table 2 illustrates the composition of the extracellular compartment calculated from the plasma values. These data are included since exchanges of ions occur between bone mineral and this fluid

TABLE 2. CALCULATED CONCENTRATION OF EXTRACELLULAR FLUID ELECTROLYTES

	Na m.eq/L	K m.eq/L	Ca^{++} m.eq/L	Cl m.eq/L	P mM/L
Control					
Mean (10)	144	3.9	1.49	109.1	2.21
SD	3.1	.38	.19	3.7	.20
Adrenalectomy					
Mean (10)	134	6.0	1.36	100.9	3.08
SD	2.6	.91	.05	3.14	.56
Prednisolone					
Mean (10)	145	3.7	1.24	106.4	1.99
SD	3.4	.49	.10	4.8	.24

rather than with the plasma. The calcium drop is magnified by the Donnan factor in the adrenalectomized and steroid treated groups: -8.7% for the former and -16.8% for the latter.

d) *Bone:* The basic and derived values for bone composition are summarized in Table 3. The values found in the control group are within the range obtained previously in this laboratory (3, 14). In the adrenalectomized group, water, total organic solids, and chloride remained at control levels, while bone sodium decreased by 18 m.eq/Kg bone ash (-5.6%). These changes are similar to those previously reported by others (10). Calcium increased by 234 mM ($+2.8\%$), phosphorus by 200 mM ($+3.4\%$) and carbonate by 73 mM ($+8\%$). In the steroid treated animals, the water content was slightly lower than in the controls, although ash weight and sodium values were identical. The most marked change in these animals was the 3.4% rise in bone calcium. This represented 279 mM/Kg bone ash. Phosphorus, carbonate, and chloride appeared to increase slightly, but none of these changes were statistically significant. The significance of the results are given as P values in Table 4 together with the per cent variations as compared with controls.

Morphology: Histological sections of bone from controls and steroid treated rats were made both from young animals weighing 100 gm and from

TABLE 3. BASIC AND DERIVED BONE DATA

	H_2O Gms/Kg	Ash %	Cl m.eq/Kg of dry FFS	Crystal Ca mM/Kg Ash	Crystal P	$\frac{Ca}{P}$	Na* m.eq/Kg Ash	Crystal† Na	$CO_3^=$ mM/Kg of dry FFS	$CO_3^=$¶ mM/Kg Ash
Control										
Mean (10)	191	73.7	16.6	8,316	5,800	1.43	371	342	858	1,164
SD	8.6	1.05	1.2	186	264		9.2	9.2	39	53
Adrenalectomy										
Mean (10)	194	74.5	16.4	8,550	6,000	1.43	357	323	931	1,250
SD	13.9	1.82	1.6	376	236		8.4	8.6	62	83
Prednisolone										
Mean (10)	185	73.5	17.1	8,595	5,970	1.44	373	341	876	1,191
SD	10.4	1.47	1.3	166	180		12.5	10.7	36	49

* Not corrected for ECF Na.
† Corrected for ECF Na.
¶ Calculated from value obtained on F.F. dry solid.

TABLE 4. % OF VARIATIONS AND p VALUES AS COMPARED WITH NORMAL CONTROLS

		Na	K	Ca*	Cl	P	$CO_3^=$
Plasma							
Adrenalectomy	%	−7.43	+58.8	−3.35	−8.1	+36	
	p	<0.001	<0.001	NS	<0.001	<0.001	
Prednisolone	%	0	−5.12	−9.5	−3.2	−12.4	
	p	NS	NS	<0.05>0.02	<0.01	<0.05>0.02	
ECF							
Adrenalectomy	%	−6.9	+53.8	−8.7	−8.3	+39.3	
	p	<0.001	<0.001	<0.1>0.05	<0.001	<0.001	
Prednisolone	%	+0.7	−5.12	−16.8	−5.8	−10	
	p	NS	NS	<0.01>0.001	<0.1>0.05	<0.05>0.02	
Bone							
Adrenalectomy	%	−5.6	+4.3	+2.8	−1.2	+3.4	+8.5
	p	<0.001	NS	NS	NS	NS	<0.01>0.001
Prednisolone	%	−0.3	−13.6	+3.4	+3.0	+2.9	+2.1
	p	NS	NS	<0.01	NS	NS	NS

* In plasma: total Ca.
In ECF: ionized Ca.

adults weighing about 300 gm. Measurements of bone calcium were made at the same time. The purpose was to ascertain whether the changes observed following prednisolone administration were similar to those reported in young rats by Follis (18) using cortisone and whether there was any morphological equivalent of the chemical findings of increased bone calcium concentration. The histological changes observed in the young rats treated with prednisolone were similar to the observations of Follis. The growth cartilage was markedly thinned and appeared to be heavily calcified. The primary spongiosa consisted of a dense network of thick trabeculae (Figs. 1 and 2). Osteoclasts were sparse. In the 300 gm rats treated with prednisolone, thinning of the growth cartilage was not obvious, but the changes in the primary spongiosa were apparent, although to a lesser degree than in the younger animals (Figs. 3 and 4).

Table 5 shows that the bone calcium concentration of the young steroid-treated animals was increased 10.3%, while in the treated adults a 3.4% rise was found when compared with their respective controls. Thus there was a parallel between the chemical and morphological findings, the most marked changes being seen in younger animals.

TABLE 5

	Young Animals		Adult Animals	
	Bone Ca mM/Kg. Ash	Morphological Changes	Bone Ca mM/Kg. Ash	Morphological Changes
Control	8,365	0	8,316	0
Prednisolone	9,225	++++	8,595	++
% increase	10.3%		3.4%	

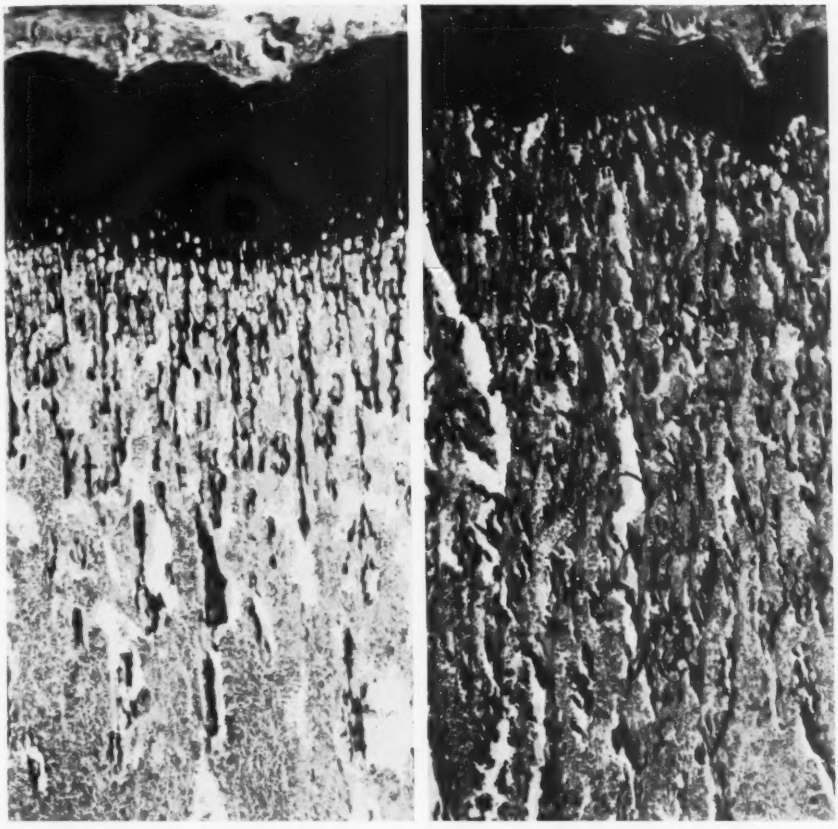

FIG. 1. (left) Untreated control rat (100 gm.): proximal tibial epiphysis. Aldehyde—fuchsin, Orange G., ×100.

FIG. 2. (right) Prednisolone-treated rat (100 gm.): proximal tibial epiphysis. Note marked thinning of epiphyseal cartilage, and note the primary spongiosa, which consists of a dense and extensive network of thick trabeculae. (cf. Fig. 1) (Aldehyde—fuchsin, Orange G., ×100.)

DISCUSSION

The changes observed in plasma and bone of adrenalectomized animals are consistent with the concept that the distribution of sodium and calcium between extracellular fluid and bone follow the physicochemical laws of solubility applicable to a two phase system of fluid and solid. The general depletion of body sodium which occurred was reflected by a 5.6% decrease in bone sodium concentration. The lack of change in plasma and bone sodium of steroid treated animals can also be fitted into this concept. In contrast, the changes in plasma and bone calcium seen in the steroid treated

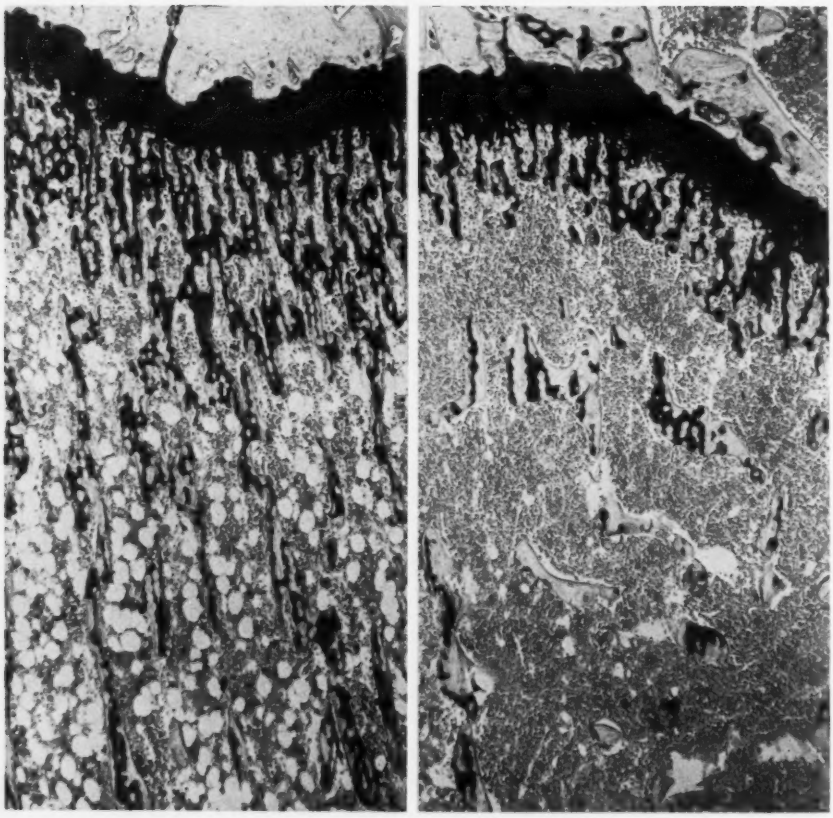

FIG. 3. (left) Prednisolone-treated rat (300 gm.): proximal tibial epiphysis. The growth cartilage is not obviously thinned, (cf. Fig. 4: untreated control) but the primary spongiosa shows changes similar to those seen in the younger (100 gm.) rats, but to a lesser degree. (cf. Fig. 2) (Aldehyde—fuchsin, Orange G., ×100.)

FIG. 4. (right) Untreated control rat (300 gm.): proximal tibial epiphysis. (Aldehyde—fuchsin, Orange G., ×100.)

group cannot be accounted for by such a simple two phase equilibrium. There was a significantly higher bone calcium concentration in these animals, despite a 16.8% decrease in the calcium concentration of the extracellular fluid.

It is therefore apparent that treatment with Prednisolone produced a situation which was by no means the opposite of adrenalectomy. Instead it was quite comparable to a state of hypoparathyroidism in which hypocalcemia occurs and bone resorption is thought to be decreased. In fact, the morphological changes seen in the bones of these animals were those which might be anticipated following parathyroidectomy (19).

Thus it might appear that Prednisolone at this dose in the rat interferes

with the action of parathyroid hormone, thus preventing the mobilization of calcium from bone to support the supersaturation of the plasma. If prednisolone enhanced the oxidation of bone citrate, as is suggested by the demonstration of decreased bone citrate following cortisone administration (20), less acid would be available to mobilize calcium from the bone mineral according to Neuman's hypothesis and bone calcium would be high as was found in these experiments. However, were this the mechanism involved Clark and Geoffroy should have found an increased uptake of Ca^{45} in the bones of their animals treated with Prednisolone, while, in fact, they observed a decreased uptake of isotope (11).

Another possibility, which is compatible with our findings and those cited above, is that the action of Prednisolone (and perhaps other glucocorticoids as well) is to reduce the permeability of the organic matrix of the bone. Since bone crystal appears to be encased in matrix (21), any exchange of ions between bone mineral and the extracellular fluid must depend on diffusion across this organic phase and therefore on its permeability. A decrease in this permeability would produce a barrier to diffusion equilibrium between the crystals of the mineral and their hydration shells and the extracellular fluid. Two results would ensue. First, calcium ions would not be able to diffuse out into the extracellular fluid thus producing a relatively high bone mineral calcium concentration, as we have shown. Second, the size of the exchangeable calcium pool in the body and the uptake of Ca^{45} by bone would be reduced. Under these circumstances, one would expect the specific activity of the urinary calcium to be increased as well as the bone uptake of isotope to be decreased, as was the case in Clark and Geoffroy's experiments. The fact that ACTH and cortisone have been shown to reduce the permeability of the ground substance of connective tissue of comparable composition in other areas of the body by increasing its polymerization lends support to this view (22).

It is apparent that the data presented are only sufficient to suggest and not to prove such a mechanism. However, they do demonstrate that, under certain conditions, the distribution of ions between bone mineral and extracellular fluid seems to be predominately dependent on local factors in the organic phase of bone which in turn may be influenced by Prednisolone and perhaps other glucocorticoids as well.

Acknowledgment

The authors wish to express their gratitude to Dr. Edward Henderson of the Schering Corporation who generously supplied the Prednisolone. They are indebted to Miss S. E. Fox for technical assistance.

REFERENCES

1. Hastings, A. B. and C. B. Huggins: *Proc. Soc. Exper. Biol. & Med.* **30**: 458. 1935.
2. Nichols, G., Jr. and N. Nichols: *Am. J. Physiol.* **186**: 383. 1956.
3. Nichols, N. and G. Nichols, Jr.: *Proc. Soc. Exper. Biol. & Med.* **96**: 835. 1957.

4. Bergstrom, W. H. and W. H. Wallace: *J. Clin. Invest.* **33:** 867. 1954.
5. Stoll, W. R. and W. F. Neuman: *J. Am. Chem. Soc.* **78:** 1585. 1956.
6. Neuman, W. F., T. Y. Toribara and B. J. Mulryan: *Am. Chem. Soc.* **78:** 4263. 1956.
7. Neuman, W. F.: Atomic Energy Report, U. R. 238, 1953.
8. Thompson, M. M.: Doctoral Thesis, Radcliffe College, Cambridge, Mass., 1956.
9. Stern, T. N., V. V. Cole, A. C. Bass and R. R. Overman: *Am. J. Physiol.* **164:** 437. 1951.
10. White, H. L. and D. Rolf: *Am. J. Physiol.* **180:** 287. 1955.
11. Clark, I. and R. F. Geoffroy: *Fed. Proc.* **16:** 165. 1957.
12. Bunim, J. J., M. M. Pechet and A. J. Bollet: *J.A.M.A.* **157:** 311. 1955.
13. Bergstrom, W. H.: *J. Biol. Chem.* **206:** 711. 1954.
14. Nichols, N. and G. Nichols, Jr.: *J. Clin. Invest.* **37:** 1676. 1958.
15. Manery, J. F.: *Physiol. Rev.* **34:** 334. 1954.
16. McLean, F. C. and A. B. Hastings: *J. Biol. Chem.* **107:** 337. 1934.
17. Clark, I., R. F. Geoffroy and W. Bowers: *Endocrinology* **64:** 849. 1959.
18. Follis, R. H., Jr.: *Proc. Soc. Exper. Biol. & Med.* **76:** 722. 1951.
19. Weinmann, J. P. and H. Sicher: Bone and Bones, Fundamentals of Bone Biology. St. Louis, The C. V. Mosby Company, 1955, page 268.
20. Harrison, H. C., H. E. Harrison and E. A. Park: *Am. J. Physiol.* **192:** 432. 1958.
21. Fitton-Jackson, S. and J. T. Randall: Bone Structure and Metabolism, Ciba Foundation Symposium, p. 47, 1956.
22. Seifter, J., W. W. Ehrich, D. H. Baeder, A. J. Butt and E. A. Hauser: *Ann. N. Y. Acad. Sci.* **56:** 693. 1953.

DECREASE IN AEROBIC GLYCOLYSIS OF ERYTHROCYTES FOLLOWING THE CONTINUOUS ADMINISTRATION OF L-TRIIODOTHYRONINE[1,2]

B. CALESNICK, V. R. ALTARELLI AND M. A. SPIRTES

Laboratory of Human Pharmacology, Hahnemann Medical College, Philadelphia, Pennsylvania

ABSTRACT

The daily intravenous administration of L-triiodothyronine, L-T_3 (125 μg./kg.), to rabbits produced biphasic effects on the aerobic glycolysis rate of erythrocytes, *in vitro*. An increase in the glycolysis rate was observed in 24 hours; the increase reached a peak in 4 days. Following this, the glycolysis rate fell off and at 21 days, when toxic effects occurred, it was only 62% of normal. The possibility that a decrease of RBC glycolysis rate might be used as a clinical laboratory test for thyrotoxicity must be considered.

IT IS well established that the peripheral cellular effects of the thyroid hormone cannot be demonstrated in all tissues (1). In some tissues, such as kidney and salivary gland, changes in anaerobic glycolysis measured *in vitro* by the Warburg technique parallel the level of thyroid activity (2–5). On the other hand, no changes in the anaerobic glycolysis have been found in the liver, spleen, or brain of hyperthyroid animals (2, 5, 6). No report has been found in the literature concerning the glycolysis of erythrocytes in thyroid diseases. In this presentation the effects of L-triiodothyronine (L-T3) on aerobic glycolysis in mammalian erythrocytes was studied in order to determine the possibility of establishing a diagnostic test for evaluating thyroid activity. This compound was found to produce a prolonged decrease in the glycolysis of RBC in rabbits and humans. Only the animal data are presented in this paper.

MATERIALS AND METHODS

Fasting blood was collected with siliconized equipment using heparin as an anticoagulant. A red cell count, white cell count, and a hematocrit were done on each sample by the usual clinical laboratory methods. The samples were centrifuged for 20 minutes at approximately 3000 rpm in a clinical angle centrifuge. The plasma was carefully withdrawn from the tube and placed in a second tube. The buffy coat of white cells was then aspirated and discarded. The lower half of the packed red cells was then

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[2] This investigation has been made with the assistance of a grant from the Committee on Research, Council on Drugs, American Medical Association.

carefully aspirated and added to an equal volume of plasma to obtain a hematocrit of 50%. The white cell count was then repeated on the reconstituted blood. Glucose determinations, in duplicate, were made by the method of Nelson (7). Samples were incubated in air in a constant temperature water bath at 37.5° C after preliminary experiments revealed no difference between the rates of glycolysis measured under aerobic and anaerobic conditions. The tubes were shaken every 10 minutes and aliquots were withdrawn at 10 and 70 minutes for glucose analyses. These periods represented the control and one hour determinations.

Animals. A group of 31 white rabbits (2.2–2.5 kg.), fed Purina Rabbit Chow containing 0.25% iodized salt was used initially to obtain the normal glycolysis rate for erythrocytes and both protein bound and total serum iodine (8) levels.

In a pilot study, 8 of these rabbits then received daily intravenous doses of 125 μg/kg. of L-T3 to determine the effects produced over a period of 14 days. The animals were weighed before medication and blood studies were performed and these procedures were repeated at the end of this experimental period. The solution used was prepared every third day by dissolving 5 mg. of L-T3 in 1.3 ml. of 0.01 N sodium hydroxide solution and diluted to a final volume of 10 ml. with normal saline. The final preparation was clear and had an average pH of 9.5. It was kept refrigerated at 5° C.

Intravenous doses of L-T3 (125 μg./kg.) were then administered daily to a second group of 16 white rabbits which acted as their own controls. All blood samples were obtained immediately prior to the administration of the individual dose of this compound. The blood was obtained daily from the left ventricle by cardiac puncture for the first four days and then weekly up to the time the animals manifested signs of thyrotoxicity. These signs appeared in 19–21 days in the form of weight loss, shedding of hair, and hyperexcitability.

Preparation of the blood samples for these studies was completed within one hour after drawing them. The glycolysis rate is expressed as mg. glucose utilized by 100 ml. packed red cells per hour.

RESULTS

The normal values obtained in the rabbits are listed in Table 1.

The results obtained in the pilot study (Table 1) revealed a decreased glycolysis rate of RBC and body weight. Total serum iodine increased while serum PBI fell insignificantly.

As can be seen in Fig. 1, the daily administration of L-T3 produced an

TABLE 1. AVERAGE VALUES OBTAINED IN NORMAL UNTREATED RABBITS AND IN RABBITS AFTER 14 DAYS OF DAILY INTRAVENOUS ADMINISTRATION OF L-TRIIODOTHYIONINE

	Control*		Treated**	
	Mean ±	S.E.	Mean ±	S.E.
Weight of Animals (kg.)	2.39	0.41	2.0	0.2
Glycolysis rate of RBC (mg. glucose utilized) per 100 ml. packed cells per hour at 37° C)	44.9	3.0	24.0	4.0
Serum PBI (μg. %)	7.7	0.1	6.4	0.4
Serum total iodine (μg. %)	14.0	0.7	36.5	6.2

* 31 Untreated animals.
** 8 of the control animals.

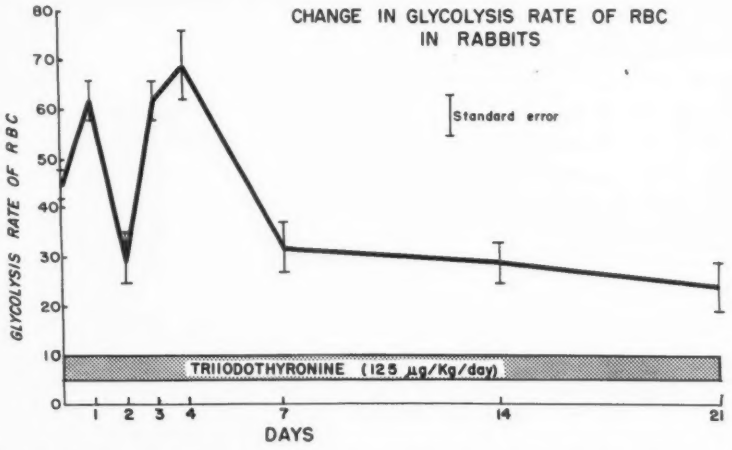

FIG. 1. *In vitro* studies conducted under aerobic conditions. Results obtained by the daily intravenous administration of L-triiodothyronine (125 µg./kg.).

increase in the glycolysis rate of RBC in 24 hours, followed by a fall at 48 hours. During the subsequent 48 hours there was a secondary, statistically significant, rise which reached its maximum at 4 days. Following this, the rate began to decline and fell to −35% of the control value at the end of the experimental period. No statistically significant change was observed in the serum protein bound iodine (Fig. 2). The total serum iodine rose slowly during the first 7 days to reach a maximum. Serum nonprotein bound iodine (total iodine minus PBI) rose abruptly during the first seven days and continued to climb slowly thereafter. There was a loss of 22% in mean body weight and a 2.6-fold increase in total serum iodine at the end of the experiment.

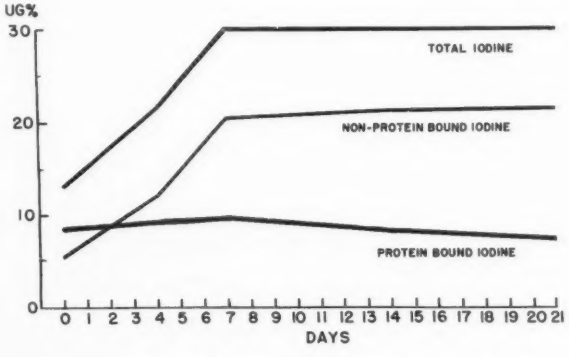

FIG. 2. Serum studies performed on rabbits. The non-protein bound iodine was calculated as the difference between total iodine and protein bound iodine.

DISCUSSION

In performing the present studies only the free and diffusible sugar was determined. Since sugar passes through the red cell surface by forming a temporary complex with a cell-surface component (9), an interference with the utilization of sugar may result from inhibition of this transport mechanism. It has been reported that thyroxine has no effect on this transport system (10). However, it is possible that continued L-T3 administration could change the RBC surface permeability, as suggested by Ermans' work (11). It might be postulated that cofactors necessary for glucose metabolism leak out to a greater degree than with normal cells and become a limiting factor for glycolysis. A study of ATP, DPN, and TPN leakage from RBC of euthyroid and dysthyroid animals is therefore indicated.

The fact that no significant change was found in serum PBI after injection of L-T3 is possibly due to two factors. First, L-T3, like thyroxin in large doses (12), suppresses TSH release and therefore thyroid hormone release into the blood. Second, it is bound to the red cells (13) and the peripheral organ cells (14) very rapidly and is not added to the measurable serum PBI. Others have also reported a failure of serum PBI to rise following the administration of L-T3 (15).

The very large increase in the "iodine pool" could possibly be due to the biotransformation of L-T3. This is suggested by the studies which revealed a deiodination of this compound (15) which is then excreted as iodide (14).

REFERENCES

1. Barker, S. B.: *Physiol. Reviews* **31**: 205. 1951.
2. McEachern, D.: *Bull. Johns Hopkins Hosp.* **56**: 145. 1935.
3. Goldstein, M. S.: *J. Biol. Chem.* **199**: 923. 1952.
4. Dresel, K.: *Klin. Wchnschr.* **7**: 504. 1928.
5. Anselmino, K. J., O. Eichler and H. Schlossmann: *Biochem. Ztschr.* **205**: 481. 1929.
6. Spirtes, M. A.: *Proc. Soc. Exper. Biol. and Med.* **93**: 386. 1956.
7. Nelson, N. D.: *J. Biol. Chem.* **153**: 375. 1944.
8. Barker, S. B. and M. J. Humphrey: *J. Clin. Invest.* **30**: 55. 1951.
9. Le Fevre, P. G.: Symposia of the Society for Experimental Biology, VIII, Active Transport and Secretion, Academic Press, Inc. pp. 118–135. 1954.
10. Wilbrandt, W.: Symposia of the Society for Experimental Biology, VIII, Active Transport and Secretion, Academic Press, Inc. pp. 136–162. 1954.
11. Ermans, A. M.: Influence de l'activité thyroidienne sur la vitesse d'éxchange du phosphore globulaire, Bruxelles, Acta Medica Belgica. 1958.
12. Beierwaltes, W. H. and G. E. Ruff: *A.M.A. Arch. Int. Med.* **101**: 569. 1958.
13. Crispell, K. R. and J. Coleman: *J. Clin. Invest.* **35**: 475. 1956.
14. Perlmutter, M. and S. Slater: *J.A.M.A.* **158**: 718. 1955.
15. Flock, E. V. and J. L. Bollman: *J. Biol. Chem.* **214**: 709. 1955.
16. Larson, F. C. and E. C. Albright: *Endocrinology* **63**: 183. 1958.

COMPARISON OF *IN VIVO* AND *IN VITRO* EFFECTS OF TRIIODOTHYRONINE[1]

ROGER L. GREIF

Department of Physiology, Cornell University Medical College, New York, New York

ABSTRACT

Thyroidectomized rats were given intravenous injections of 15 mg. of labeled 3,5,3′-L-triiodothyronine after removal of one kidney. The second kidney was removed 10 minutes later, and Q_{O_2} values of slices from the injected kidney were compared to those of the control kidney slices to which hormone was added *in vitro*. The maximum Q_{O_2} obtainable with added hormone 72 hours after removal of the tissue, with interim refrigeration, was $1.59 \pm .17$. The Q_{O_2} of slices from the injected kidney at this time was $2.75 \pm .11$. Neither tissue hormone concentration nor chromatographic demonstration of different compounds explain the greater Q_{O_2} from *in vivo* administration, which can be diminished or entirely eliminated by removing the second kidney 30 seconds after injection or by ligating hepatic blood vessels prior to the start of the experiment.

THE *in vitro* effects of thyroid hormones upon the oxygen consumption (Q_{O_2}) of slices of excised rat kidney have been described by Barker (1). We have noted that thyroid hormone induced delay in the fall in Q_{O_2} which normally follows tissue removal is present whether the hormone reaches the kidney slice by *in vitro* addition or if it is injected intravenously before excision of the kidney. When the tissue is supplied with hormone via the vascular system, the addition of more hormone *in vitro* produces a further effect on the Q_{O_2} (2). The present experiments were designed to determine whether the tissue response is similar if large amounts of hormone are added to the kidney slice *in vitro* or reach the kidney via the vascular system prior to excision.

PROCEDURE

The experiments were carried out as previously described (2). As a control, one kidney was removed from a thyroidectomized rat (average weight, 300 gm.) under ether anesthesia; then the hormone was injected into the inferior vena cava. Each rat received 15 mg. of 3,5,3′-L-triiodothyronine[2] dissolved in .2 N sodium hydroxide and made up to a volume of 1 ml. with .85% saline. Tracer amounts of I^{131} labeled triiodothyronine[3] were added to the hormone prior to injection, and tissue radioactivity was measured by suspending the weighed slices in cold buffer in counting tubes before the initial transfer

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[2] Generously provided by Dr. A. E. Heming of the Smith, Kline, and French Co.

[3] Abbott Laboratories, Oak Ridge, Tennessee.

to Warburg vessels. The buffer contained .12 M NaCl, .003 M KCl, .0012 M $MgSO_4$, .017 M D,L-alanine (pH 7.8) and .01 M glucose. Final pH was 7.2. When labeled triiodothyronine was added *in vitro*, the slices were removed from the vessels for counting after 70 minutes at 37° C, and were then replaced in the original vessesl for storage at 4° C. The time intervals between injection of triiodothyronine and removal of the second kidney are indicated with the individual experiments.

Liver ligation was carried out by passing a loop of heavy braided surgical silk around as much of the organ as possible, and drawing the loop tight. This ligation was done before removal of the control kidney, and compressed both the hepatic veins and intrahepatic portions of the portal veins.

Tissue extracts were made by homogenizing slices in cold absolute ethanol and centrifuging down the precipitate. The supernatant fluid was concentrated by evaporation in air at room temperature and applied to Whatman #1 filter paper. Ascending chromatograms were developed in n butanol-dioxane-ammonium hydroxide, butanol acetic acid, and tertiary amyl alcohol-ammonium hydroxide systems (3). The paper was air dried and placed in contact with "No Screen" X-Ray film for the appropriate exposure time. Unlabeled thyroxine, triiodothyronine, diiodotyrosine, and potassium iodide were applied to each chromatogram, and their position subsequently located with diazotized sulfanilic acid spray and palladium chloride spray.

RESULTS

Maximum tissue response to in vitro hormone addition

Figure 1 shows the effect upon the Q_{O_2} of rat kidney slices of adding increasing amounts of triiodothyronine to the vessels. The mean of between 11 and 28 separate vessels is represented by each point, and the standard deviation about the mean is also shown. The Q_{O_2} values 48 hours and 72 hours after the start of the experiment are indicated by the open circles and the triangles respectively. Values for the 24-hour and immediate oxygen consumption measurements are not included because in no instance do

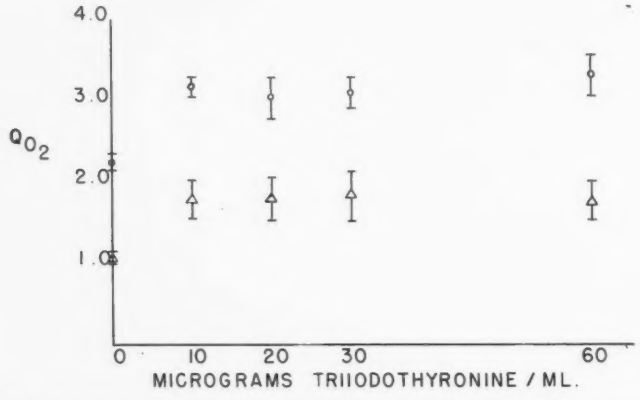

FIG. 1. Effect of increasing T3 concentration *in vitro*. Circles show mean Q_{O_2} values 48 hours after start of experiment, triangles the values after 72 hours. Standard deviation of each value is indicated.

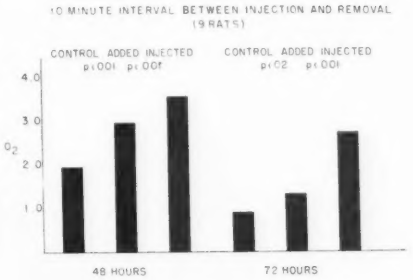

FIG. 2. The mean Q_{O_2} values for slices from control kidney, control kidney with added triiodothyronine 10 μ/ml., and slices from the injected kidney are shown as columns and significance of the difference in height of the columns is placed between the columns being compared. Ten minute interval between hormone administration and removal of injected kidney.

triiodothyronine effects show up prior to 48 hours when large amounts of hormone are used. It can be seen that there is no significant increase in Q_{O_2} obtained by the addition of more than 10 μg. of hormone per ml. of buffer, so that this amount was routinely used in subsequent *in vitro* studies.

Tissue response ten minutes after intravenous hormone injection

Figure 2 shows the results obtained when the Q_{O_2} of slices of the control kidney and of control kidney with added triiodothyronine are compared to the Q_{O_2} of slices from the injected kidney. The probability values are placed between the columns which are being compared statistically. It can be seen that 48 hours after the start of the experiment there is a much greater tissue response to the injected hormone than to the *in vitro* addition of hormone, and that the difference is even more striking at 72 hours. Additional hormone added *in vitro* to the vessels containing slices from the injected kidney produced no effect on the Q_{O_2}.

Tissue response 30 seconds after hormone injection

In this group of animals the interval between injection and the removal of the injected kidney was shortened to 30 seconds. Figure 3 shows the results obtained, with statistical evaluation. It can be seen that the difference between the Q_{O_2} values with triiodothyronine added *in vitro* and injected *in vivo* is no longer present. The *in vitro* addition of triiodothyronine to slices from the injected kidney did not produce a significant further in-

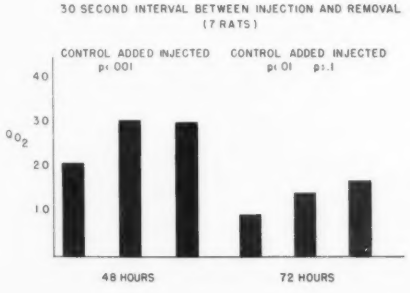

FIG. 3. Effect of shortening interval between hormone injection and removal of injected kidney to 30 seconds. For legend, see Fig. 2.

crease in Q_{O_2} after 48 hours, but when compared to the control slices suspended in buffer containing triiodothyronine, 10 μg. per ml., there was an effect at 72 hours significant at the 2% level.

Tissue response to hormone after liver ligation

When all of the hepatic tissue that could be conveniently passed through a loop of braided silk was excluded from the circulation, the results, as seen in Figure 4, showed that the difference between the Q_{O_2} of the control kidney with added triiodothyronine, removed after ligation, and the injected kidney, removed 10 minutes after triiodothyronine administration decreased to insignificant levels after 48 hours, and was significant at the 5% level after 72 hours. *In vitro* addition of triiodothyronine to slices of injected kidney produced a further rise in Q_{O_2}, which was not significant when compared to such slices suspended in buffer alone, but was higher than the Q_{O_2} of slices of the control kidney plus *in vitro* triiodothyronine at the 1% level of significance.

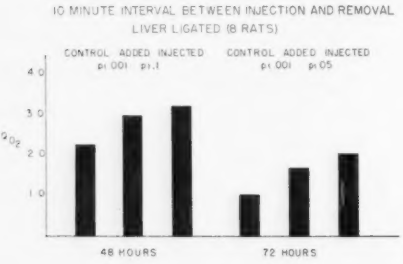

FIG. 4. 10 minute interval between hormone injection and removal of injected kidney is maintained, but liver has been ligated. For legend, see Fig. 2.

Effect of solvent injection on the tissue response to added triiodothyronine

This proved to be negligible in every instance, whether the liver was intact or ligated. Diiodotyrosine failed to affect the Q_{O_2} of the kidney when injected intravenously in our previous study (2).

Concentration of labeled material in tissue

Figure 5 compares the concentration of labeled material in slices taken from flasks to which 60 μg. of hormone per ml. of buffer had been added to the concentration reached after intravenous injection under the different experimental conditions. It should be noted that the highest tissue concentration is found in slices made from the kidneys of the rats with ligated livers. The differences in height of the columns are all statistically significant.

Nature of the I^{131}-containing compounds in tissue

Chromatographic examination was made of ethanol extracts from slices of kidney subjected to the different experimental conditions. In all in-

stances over 75% of the radioactivity was found in the triiodothyronine spot. There were some spots which are still unidentified, most marked after incubation for 48 hours. Triiodothyroacetic acid and iodide appeared to be present on these chromatograms, and a spot was present in the approximate position of a glucuronic acid conjugate. On the basis of the chromatograms and radioautographs, it has not been possible to distinguish between the extracts made from tissues incubated for 48 hours with and without liver ligation, nor did there appear to be any difference between extracts made from kidney removed 30 seconds after injection and 10 minutes after injection which were extracted prior to incubation.

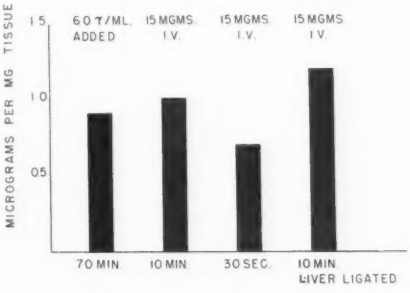

FIG. 5. Concentration of labelled material in tissue based upon I^{131} measurement. Columns show concentration of labeled material in slices of injected kidney prior to measurement of Q_{O_2}. In the case of added triiodothyronine, measurements are made on slices removed from vessels after 70 minutes in contact with hormone at 37° C.

DISCUSSION

As contrasted to the results of our study using relatively low tissue triiodothyronine concentration (2), the present findings show that if sufficient hormone reaches the tissue via the vascular system, a significantly greater Q_{O_2} response can be obtained than the maximum we have observed *in vitro*. The difference between the two means of supplying the tissue with hormone can either be reduced or eliminated by shortening the time between injection and removal of the kidney, or by excluding the liver from the circulation.

The simplest explanation for the difference would be that higher concentrations of hormone are found in tissue supplied by the vascular system. Figure 5, however, shows that the highest tissue I^{131} concentration is found in the group of animals with ligated livers, an observation similar to that of Flock and Bollman (4) using eviscerated rats. This group of animals showed a small response to injected triiodothyronine. Furthermore, the *in vitro* addition of more triiodothyronine to such tissues, perfused with hormone *in vivo*, did not usually produce an increased Q_{O_2}.

The experiment involving shortening the period between hormone injection and tissue removal and the experiment with liver ligation were designed to test the hypothesis that some metabolic transformation had occurred during the ten minutes between hormone injection and removal of

the second kidney, and that the liver was the principal site of the transformation. Although the results favor this hypothesis, it should be noted first that the tissue I^{131} concentration is lower 30 seconds after injection than 10 minutes after injection, so that this in itself might be at least a partial explanation of the difference in Q_{O_2} between these groups. In the case of liver ligation, it should also be noted that this is a procedure involving acute reduction in blood volume, with many hormonal and biochemical changes, and before assigning an important role to the liver in "activating" thyroid hormone, further studies should be carried out upon animals with intact livers but blood volumes reduced by controlled hemorrhage. The reverse experiment, with liver damage and normal blood volume, might also be tested.

If a metabolic transformation does occur, it need not be in the thyronine derivative itself. The possibility exists of some protein-hormone combination similar to that postulated for estrogens (5). Our chromatograms, which resemble those obtained with *in vitro* hormone by Etling and Barker (6), could not detect such a complex. Our finding of a maximum tissue response to added triiodothyronine (Fig. 1) is also a confirmation of Barker's results (7).

Finally, it should be emphasized again that the entire system under study in the present experiments is an artificial one which measures the effect of large amounts of thyroid hormones in preserving a failing system. An actual increase in Q_{O_2} above the control level in slices of excised tissue produced by thyroid hormones has yet to be demonstrated.

Acknowledgment

The author is indebted to Jean Moroney for expert technical assistance.

REFERENCES

1. Barker, S. B.: *Endocrinology* **59**: 719. 1956.
2. Greif, R. L. and J. Moroney: *Endocrinology* **64**: 937. 1959.
3. Taurog, A. in Colowick, S. P. and N. O. Kaplan: *Methods in Enzymology*, Academic Press, New York. Volume IV, page 873, 1957.
4. Flock, E. V. and J. W. Bollman: *J. Biol. Chem.* **214**: 709. 1955.
5. Szego, C. M. and S. Roberts: *Endocrinology* **36**: 104. 1945.
6. Etling, N. and S. B. Barker: *Endocrinology* **64**: 753. 1959.
7. Barker, S. B.: *Ciba Foundation Colloquia on Endocrinology* **10**: 253. 1957.

A NEW METHOD FOR THE BIOASSAY OF THE CALCIUM MOBILIZING FRACTION OF PARATHYROID EXTRACTS

IRWIN CLARK,[1] WILLIAM BOWERS AND RAYMOND GEOFFROY

Merck Institute for Therapeutic Research, Rahway, New Jersey

ABSTRACT

A bioassay for the calcium mobilizing fraction of parathyroid gland extract has been developed using intact rats. This assay is based on the ability of such an extract to promote the withdrawal of radiocalcium from the skeletons of rats which had been injected with radiocalcium at least 40 days before. This procedure meets the requirements of a satisfactory assay in that: (a) The response increases with increasing dose. (b) The response is linear over a particular range of doses when expressed as log dose. (c) The variation among responses at a single dose level is independent of the magnitude of the average response at that dose.

Using this technique as little as 4 units of U.S.P. Parathyroid Extract (Lilly)/100 gm. body weight can be detected in the intact rat. The calcium mobilizing fraction is effective in the rat via the intravenous, intraperitoneal and subcutaneous routes but not by mouth.

EXTRACTS of the parathyroid gland capable of raising the serum calcium levels in experimental animals have been prepared as early as 1925 (1, 2). However, to date, an unequivocally pure hormone has not been isolated, although recently samples with activities as high as 500 units/mg. protein have been reported (3). A major handicap to isolation of the pure principle has been the lack of a simple, rapid bioassay procedure. The dog assay procedure (the standard U.S.P. method) is time consuming and requires a large dog colony (4). The two most commonly used methods at present are those of Munson (5) and Davies and Gordon (6). Both employ the parathyroidectomized rat as the test animal. Although parathyroidectomy in the rat is not a difficult operation when an electrocautery is used, it does require considerable time and skill. In addition, removal of the parathyroid glands results in damage to the thyroid gland, the significance of which is not known. In one method (5) a single heart puncture is employed, whereas two are required in the other procedure (6). The heart punctures and the individual analytical calcium determinations are also time consuming. Furthermore, it has been shown that the time of the bleeding is critical (7).

Obviously, any procedure that does not require parathyroidectomy,

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[1] Present Address: The Departments of Orthopedic Surgery and Biochemistry, College of Physicians and Surgeons, Columbia University.

heart punctures and analytical calcium determinations would be of advantage in routine testing of large numbers of parathyroid extract fractions. In the method to be described, all of these operations have been eliminated.

The direct action of parathyroid extracts upon bone has been amply documented by many different techniques (8–18). Singer and Armstrong (19) have shown that approximately 52 days after the injection of radiocalcium, the isotope is distributed almost entirely within the "stable" fraction of bone. Furthermore, it has been shown that parathyroid extracts can mobilize this radiocalcium from this "stable" bone fraction (17, 18).

The assay method to be reported here is based on the ability of parathyroid extracts to mobilize radiocalcium from "stable" bone in intact rats.

MATERIAL AND METHODS

Male Holtzman rats weighing between 120 and 150 gm. were fed a calcium-free diet for 3 days. Each rat then received 100 μc. of Ca^{45} in the form of $CaCl_2$. It was hoped that by maintaining the rats on a calcium-free diet (20), a more uniform uptake of isotope by each rat might be achieved. After injection of the isotope, the animals were maintained on a Purina chow diet for at least 45 days. The rats then received the calcium-free diet for 5 days, during which time they were kept in individual cages. The following day, the appropriate dose of U.S.P. Parathyroid Extract (Lilly) was administered to rats maintained in individual metabolism cages designed to separate urine from feces. During the urine collection period, all rats received 10% glucose in their drinking water to insure adequate urine volumes. In the experiments comparing the different routes of administration of Lilly extract on urinary radiocalcium, individual urines were collected at 3, 6, 9 and 24 hours. During the development of the assay and in the assays themselves, individual 24-hour pre-treatment and 24-hour post-treatment urines were collected.

In earlier experiments, suitable aliquots of filtered urine were plated in stainless steel planchets, air dried overnight, and dried in an oven at 80° C for 24 hours. Samples were then counted in a windowless counter long enough to insure a statistical counting error of not more than $\pm 2.5\%$ and in most cases not over $\pm 1\%$. Suitable corrections were made for self absorption and background. In later experiments the counting procedure was changed. The calcium in an aliquot of urine was quantitatively precipitated as the oxalate according to the procedure of Lutwak (21). It was then dissolved in 0.3 ml. of dilute HCl. Then, 6.0 ml. of alcohol and 13.0 ml. of toluene, which contained 3% 2,5-diphenyloxazole (DPO), and 1% p-Bis-2-(5-phenyloxazole)-benzene (POPOP), were added and the mixture was counted in a Packard liquid scintillation counter.

RESULTS

Comparison of the effects of Lilly's Parathyroid Extract following different routes of administration

Figure 1 shows the amounts of radioactive calcium excreted during each collection period, and Figure 2 shows the cumulative excretion of isotope throughout the 24-hour period. The earliest increase in urinary Ca^{45} was seen after intravenous injection where maximum excretion was reached at about 6 hours and then declined rapidly. After intraperitoneal injection an increased excretion was also evident at 3 hours but the maximum was not reached until about 9 hours, after which time there was a decline. After

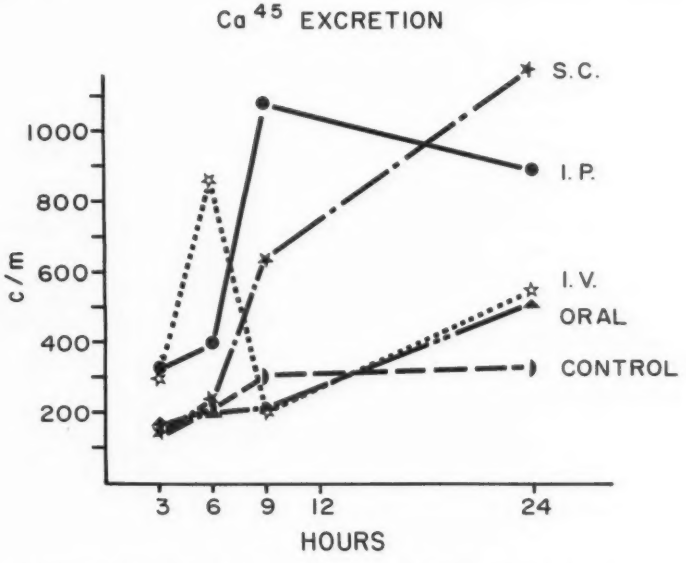

FIG. 1. Average urinary Ca^{45} following the administration of U.S.P. Parathyroid Extract (Lilly) by different routes.

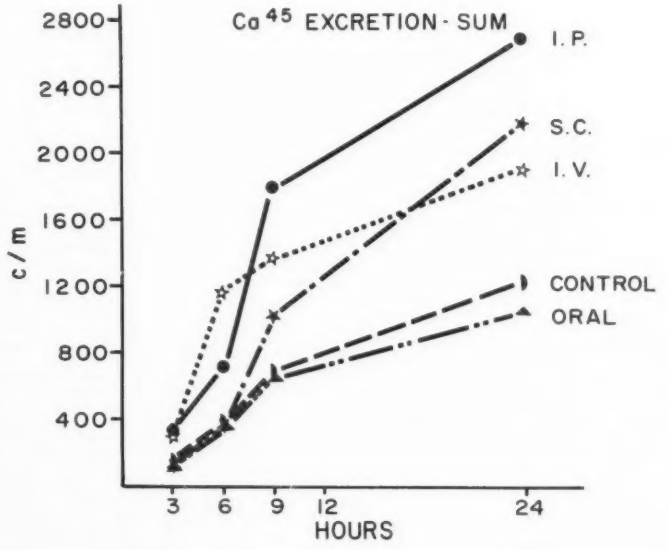

FIG. 2. Average cumulative urinary Ca^{45} following the administration of U.S.P. Parathyroid Extract (Lilly) by different routes.

subcutaneous injection, a significant increase was not evident until the ninth hour. The 24 hour figure was even larger. However, since there were no collections during the 9–24 hour period, the peak excretion was probably between these two times. Although a greater amount of radioactivity was excreted after intraperitoneal injection, it was found that greater reproducibility from experiment to experiment could be obtained when the subcutaneous route was used.

Effect of increasing amounts of U.S.P. Parathyroid Extract (Lilly) upon urinary radiocalcium

As can be seen in Table 1, increasing amounts of the extract resulted in excretion of progressively larger amounts of Ca^{45} in the urine. Moreover, a

TABLE 1. AVERAGE URINARY Ca^{45} FOLLOWING INCREASING DOSES OF LILLY'S PARATHYROID EXTRACT

Treatment	No. of animals	Average total 24-hour urinary Ca^{45}		
		Pre-treatment	Post-treatment	Difference
		(Counts/Min.)		
Controls	6	13,300	13,000	−300
2 Units*/100 gm., s.c	6	11,300	26,300	+15,000
4 Units*/100 gm., s.c.	6	10,500	35,300	+24,800
8 Units*/100 gm., s.c.	6	12,000	34,000	+22,000
16 Units*/100 gm., s.c.	6	9,300	48,500	+39,200
32 Units*/100 gm., s.c.	6	11,300	61,700	+50,400
48 Ynits*/100 gm., s.c.	6	10,400	84,600	+74,200

* U.S.P. Parathyroid Extract (Lilly).

good response was obtained with as little as 4 units/100 gm. Although in this and other experiments a response was obtained with 2 units, frequently there was no detectable increase at this level.

Assay procedure

It was observed that although each rat originally received the same amount of Ca^{45}/100 gm. body weight, after 50 days the excretion of the isotope by the individual rats varied. To minimize this variation the radioactivity value of a control 24-hour pre-treatment urine was subtracted from that of a 24-hour post-treatment urine. The difference between the two was taken as a measure of hormonal activity.

In the course of preliminary investigations, doses of 2, 4, 8, 16, 32, and 64 units/100 gm. body weight were tested. While the average difference in Ca^{45} excretion increased with increasing dose, it was noted that the variation among values observed as a single dose also increased. In fact, the ratio of standard deviation to mean was approximately constant. Since this is not a desirable condition for a good assay, a transformation of the data was necessary. The use of logarithms was appropriate in this case. This transformation made a section of the log dose response curve linear and it was within this linear range of values (8, 16, and 32 units) that further assays were performed.

TABLE 2. ANALYSIS OF SIXTEEN INDIVIDUAL ASSAYS† (95% Confidence Limits)

Assay No.	Slope*	Limits About Slope	Assay** Error (S.E.)	Error/ Slope***
21	.98	± .43	.20	.21
22	.97	± .46	.22	.23
23	1.16	± .45	.22	.19
24	.32	± .38	.18	.58
25	.91	± .60	.29	.32
26	.38	± .57	.28	.74
27	.60	± .60	.24	.41
28	.68	± .45	.21	.31
+29	.71	± .40	.24	.33
+29S	.72	± .45	.28	.39
+30	1.19	± .54	.26	.22
+30S	1.03	± .51	.25	.24
32	.70	± .80	.39	.56
35S	1.08	± .73	.35	.32
36S	.70	± .45	.21	.30
37S	.68	± .60	.26	.38

* Increase in the difference of log counts/min./10-fold increase in dose of extract.
** In terms of log counts/min. (for total 24-hour urinary radiocalcium excretion).
*** Error/Slope = Index of Precision.
+ = These assays were excluded from the analysis of variance in Table 3, since 9 animals were used instead of the usual 6/dose level of extract.
† Three dose levels of standards—8, 16, and 32 units—were used in all assays.

A series of 16 assays have been analyzed. In 5 of these, radioactivity determinations were made with the windowless gas counter. In the other 11, the scintillation counter was used. The analysis of complete individual assays is seen in Table 2, and of 12 combined assays in Table 3. In all the assays three dose levels of standard were used.

DISCUSSION

Ideally, only the regression component of variation should be significant (and highly so) for a good assay, and this is the case here (Table 3). In addition, both the method and assay components are significant. In the analysis of the 16 experiments, the combined slope $=0.82$, the combined error $=0.25$ and the index of precision $=0.33$. This index of precision is not greatly different from that (0.27) reported by Munson (5).

Although no estimation of error would be possible, the analyses of pooled urines would be adequate for a rough estimation of potencies of extracts.

TABLE 3. ANALYSIS OF VARIANCE OF LOG COUNTS/MIN. IN TWELVE ASSAYS

Nature of Variation	Degrees of Freedom	Mean Square
Methods (of counting)	1	42.607*
Assays	11	1.273*
Regression	1	9.423*
Quadratic	1	.006
Regression × Assays	11	.095
Quadratic × Assays	11	.085
Regression × Method	1	.008
Quadratic × Method	1	.015
Error	176	.071

* Significant at <0.1% probability level.

In addition, the analyses of post-treatment urines are useful for qualitative and rough quantitative estimations (Table 1).

An assay using this procedure of some material isolated from bovine parathyroid gland by E. Rickes of Merck & Co., gave a value of 18 units/mg. The same material which was kindly assayed by Dr. P. L. Munson of the Harvard School of Dental Medicine using his established procedure (5) gave a value of 17.2 units/mg. Although no conclusions may be drawn from this single comparison, it is interesting that the potency of an extract measured by two different methods is in such good agreement.

The use of intact rats and the simplicity of radioactivity determinations using an automatic sample changer appear to offer some advantages over current methods for bioassay of parathyroid extracts. In addition, the 24-hour urine sample reflects changes in serum values and avoids the possibility of missing a peak increment in the serum calcium value. A disadvantage of this method is the necessity of housing isotopically labeled rats for 50 days before using them for assay purposes. However, if space is available, this poses no problem.

Acknowledgment

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REFERENCES

1. Hanson, A. M.: *Proc. Soc. Exper. Biol. and Med.* **22**: 560. 1925.
2. Collip, J. B.: *J. Biol. Chem.* **63**: 395. 1925.
3. Munson, P. L.: *Fed. Proc.* **18**: 291. 1959.
4. U. S. Pharmacopeia, 14th Edition, 1950.
5. Munson, P. L.: *Annals N. Y. Acad. of Sciences* **60**: 776. 1955.
6. Davies, B. M. A. and A. H. Gordon: *Biochem. J.* **61**: 646. 1955.
7. Rasmussen, H. and R. C. Westall: *Nature* **178**: 1173. 1956.
8. Engel, M. B.: *Arch. Path.* **53**: 339. 1952.
9. Engel, M. B., H. R. Catchpole and N. R. Joseph: *Trans. 5th Josiah Macy, Jr. Conf. on Metabolic Interrelations*: 105–129. 1954.
10. Carnes, W. H.: *Am. J. Path.* **26**: 736. 1950.
11. Heller, M., F. C. McLean and F. C. Bloom: *Am. J. Anat.* **87**: 315. 1950.
12. Stewart, C. S. and H. F. Bowen: *Endocrinology* **88**: 568. 1951.
13. Talmage, R. V., F. W. Kraintz, R. C. Frost and L. Kraintz: *Endocrinology* **52**: 318. 1953.
14. Barnicot, N. A.: *J. Anat.* **82**: 233. 1948.
15. Chang, H.: *Anat. Record* **106**: 100. 1950.
16. Chang, H.: *Anat. Record* **111**: 23. 1951.
17. Wood, K. R. and W. D. Armstrong: *Proc. Soc. Exper. Biol. and Med.* **91**: 255. 1956.
18. Clark, I. and R. Geoffroy: *J. Biol. Chem.* **233**: 203. 1958.
19. Singer, L. and W. D. Armstrong: *Proc. Soc. Exper. Biol. and Med.* **76**: 229. 1951.
20. Munson, P. L., O. A. Iseri, L. W. Parker and R. O. Greep: *J. Dental Research* **31**: 463. 1952.
21. Lutwak, L. (Personal Communication).

CORTISONE INDUCED MODIFICATIONS IN THE DEVELOPMENT OF THE CHICK EMBRYO[1]

MALKA H. MOSCONA[2] AND DAVID A. KARNOFSKY

Embryology Section, Division of Experimental Chemotherapy
Sloan-Kettering Institute for Cancer Research, New York

ABSTRACT

Single doses of cortisone acetate, applied to the allantoic vesicle or chorioallantoic membrane of the chick embryo, on the 3rd to 8th day of incubation produce a variety of disturbances in the embryo depending on the dosage and the time of injection. Cortisone is relatively more toxic to the early embryo, and in those that survive, weight inhibition, defects in the facial bones and tibia, and exteriorized viscera occur. In embryos treated at a later stage, weight inhibition is more severe, but the defects in the facial bones are no longer present, feather inhibition is marked, and there are major disturbances in osseous development. The susceptibility of various organs in the chick embryo to cortisone is specific, and varies with their stage of development.

IT HAS been found that various adrenocortical hormones, when injected into the yolk sac, inhibited the growth of the chick embryo (1–8). When cortisone acetate was injected, in large doses, into the yolk sac at 4 days of incubation, it caused, in addition to dwarfing, various developmental defects involving the eyes, feathers and skeletal system (2). The injection of cortisone onto the chorioallantoic membrane at 8 days produced teratogenic effects similar to those caused by yolk sac injection, but by the former route the steroid was about 3 times more active by weight. Cortisone, applied to the embryonic membrane at 4 days, was considerably more toxic, but in the surviving embryos some evidences of the cortisone effect were found. The accumulated data suggested that the "cortisone effect" on the embryo, in terms of growth inhibition and developmental defects, was initiated in its conspicuous form on the 8–10th day of development irrespective of when the drug was administered before this (2). There were suggestions, however, that cortisone also caused less severe, but definite effects when given earlier in development.

The production of developmental disturbances by cortisone in relation to the normal morphogenetic processes going on in the affected organs at the time of treatment has not been studied previously. In this study, an

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[2] Present address: University of Chicago, Department of Zoology, Chicago, Illinois.

attempt has been made to correlate the stage of embryonic development at the time of cortisone treatment with the resulting pattern of abnormalities. A close study of the developmental events involved in the occurrence of the cortisone-induced defects may contribute to our knowledge of normal developmental mechanisms as well as the locus of action of cortisone.

MATERIALS AND METHODS

Application of cortisone acetate. Fertile white Leghorn eggs, obtained from a commercial source, were incubated at 38° C. Cortisone acetate was available in an aqueous suspension (containing 0.9% benzyl alcohol as a preservative) in a concentration of 25 mg./1 cc. This stock suspension was suitably diluted with saline to obtain the desired concentration of cortisone.[3] The hormone was applied through a small window cut in the shell over the embryo. On the 3rd and 4th day, cortisone was dropped on the allantoic vesicle. At later stages cortisone was applied to the chorioallantoic membrane (CAM). The usual volume applied was 0.1 cc. The opening in the shell was sealed with scotch tape.

METHODS OF OBSERVATION:

Gross examination. From each batch of eggs, prior to treatment, 10 embryos were sacrificed and their developmental stages established according to Hamburger and Hamilton (9). During the experiments the eggs were candled daily and deaths recorded. Embryos were sacrificed at regular intervals till the 18th day of development.

The sacrificed embryos were first weighed and examined grossly for external abnormalities. Embryos from 14 days on were staged for "cortisone effects" according to the method described by Karnofsky, *et al.* (2). Embryos graded as 4+ had most of the characteristic effects caused by cortisone.

Special preparations. Most of the examined embryos were fixed and stored in 10% neutral formalin for further examination. A few were fixed in 95% alcohol to enable clearing and bone staining.

In two experiments femurs were dissected out, measured, and then fixed in Zenker's acid solution for histologic examination. The following methods were used for staining the skeleton.

Bone stain. Embryos were fixed in 95% alcohol and transferred to acetone to extract the fat. They were cleared in 2% KOH and stained in alizarin red. After staining the embryos were passed through rising concentrations of glycerin in water and stored in glycerin.

Cartilage stain. Embryos were fixed in 10% neutral formalin for 24 hours. They were then transferred to acid alcohol for 2 days, and then stained in 1% methylene blue in acid alcohol. After destaining of the soft tissues in acid alcohol the embryos were dehydrated, cleared in benzene and stored in methyl salicylate.

Skin whole-mounts. Embryos were fixed in formalin for 48 hours. The skin was removed, stained in very dilute aqueous hematoxylin, dehydrated, cleared in xylene and mounted in a mounting medium.

Histology. Tissues for histologic examination were fixed in Zenker's acid solution. Serial sections were made at 8 microns thickness. Most of these were stained in Ehrlich hematoxylin and Biebrich scarlet.

RESULTS

Dosage and toxicity. The dose of cortisone tolerated by embryos increased with their age at the time of injection. The overall mortality percentage

[3] Cortisone acetate will be referred to as cortisone in this report.

TABLE 1. MORTALITY PATTERN OF 200 CHICK EMBRYOS TREATED WITH 2 MG. CORTISONE ACETATE, APPLIED TO THE ALLANTOIC VESICLE ON THE 4TH DAY OF INCUBATION

	Age of embryo (days) from onset of incubation							
	6	7	8	9	10	11	12	13
No. Surviving Embryos	200	167	—	108	104	46	24	17
No. Dead Embryos	33	59	—	4	58	22	7	4
Per Cent Mortality of Embryos Injected at 4 Days	16.5	29.5	—	2	29	11	3.5	2
Cumulative Total Per Cent Deaths	16.5	46.0		48	77	88	91.5	93.5

(10 days after treatment) of embryos injected with 2 mg./egg on the all lantoic vesicle at 4 days was about 90 to 95%. It gradually decreased until it reached 24 % when injected on the 8th day CAM.

The high mortality among embryos injected at 4 days could not be attributed to an acute lethal effect of the drug, since deaths occurred at peak periods on the 7th, 10th and 11th days of incubation (3 and 6–7 days after injection) (Table 1). Table 2 summarizes the mortality figures (up to the 14th day of incubation) when single doses of 1.0 and 1.5 mg. were given to groups of embryos at 3–8 days of incubation.

Description of abnormalities. The abnormalities which resulted from treatment of embryos with cortisone varied with the age of the embryos at the time of treatment. Consequently, two separate "syndromes" could be distinguished: syndrome I was characteristic of embryos treated at 3–5 days of incubation; syndrome II occurred in embryos treated on the 6th–10th day, although occasionally embryos treated earlier showed some of its signs. There was thus an overlapping between the various manifestations

TABLE 2. EFFECTS OF SINGLE DOSES OF CORTISONE ACETATE, 1.0 AND 1.5 MG./EGG, APPLIED TO THE ALLANTOIC VESICLE OR CAM, AT 3 TO 8 DAYS OF INCUBATION, ON EMBRYONIC MORTALITY AND DEVELOPMENT

Age embryo when treated, days	Mortality at 14 days incubation		Abnormal survivors at 14 days		Facial coloboma, cleft palate No.	4+ cortisone effect No.	Exteriorized viscera No.
	No. dead / No. treated	%	No.	%			
			1.5 mg./Egg				
4	39/50	78	7	68	5	1	5
5	39/50	78	10	91	2	5	8
6	17/30	57	13	100	0	9	12
7	5/30	18	25	100	0	23	23
8	6/30	20	20	87	0	20	6
			1.0 mg./Egg				
3	70/80	88	2	20	1	0	2
4	38/50	78	7	68	5	0	7
5	35/50	70	12	80	2	2	12
6	9/30	30	17	81	0	5	17
7	5/30	18	19	76	0	14	19
8	3/30	10	20	78	0	20	4

of these syndromes, yet the abnormalities could readily be grouped into these two categories and will be described accordingly. (Fig. 1)

Syndrome I (embryos treated at 3–5 days). Embryos treated with cortisone during this period of development, when examined on the 14th–18th day, proved to be smaller in size and lower in weight than controls (Figs. 2, 4). As shown before (2), this inhibition was not as severe as that pro-

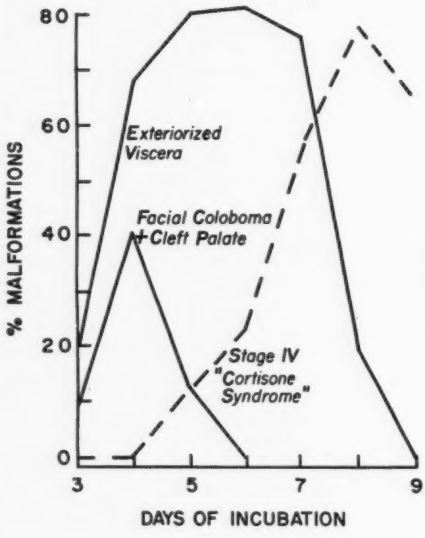


FIG. 1. The incidence of malformations among embryos surviving 14 days or longer after single doses of cortisone acetate, 1 mg./egg, applied to the allantoic vesicle or CAM at 3 to 9 days of incubation.

duced when the same dose was applied on the 8th day (Fig. 5). The morphological defects comprising this syndrome affected the beak, the extremities and the ventral body wall.

Beak malformations. The beak malformations were facial coloboma combined with cleft palate and occasionally a slightly shortened lower beak (Figs. 9, 14). In most of the cases the coloboma was bilateral, but occasionally a unilateral coloboma, combined with a cleft palate was observed; in such cases the beak was crossed towards the colobomatous side. These defects were brought about by changes in size, shape and orientation of several membranous bones of the upper beak, as may be seen in Figures 9, 11 and 14. The premaxillae (PM) developed in their appropriate position but their lateral extensions were considerably shortened. The maxillae (M) formed in a position posterior to that which they normally assume; they were smaller and their anterior extensions were missing; there was thus no continuity and contact between the premaxillae and the maxillae and they were separated by a gap. The nasal bones (N) (Fig. 14), which normally curve anteriorly, curved here in a posterior direction to join the reduced and displaced maxillae. The palatal bones (P) were considerably shortened

and flattened and, instead of extending anteriorly to join the premaxillae, they were directed sideways and met the maxillae (Fig. 11).

The incidence of facial coloboma and cleft palate depended on the developmental stage of the embryos treated and the dosages applied (Table 2, Fig. 1). With all the effective dosages, the peak incidence of these malformations (50%) was found in embryos which had been treated at 4 days

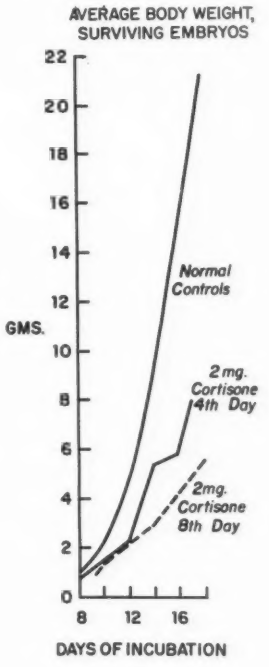
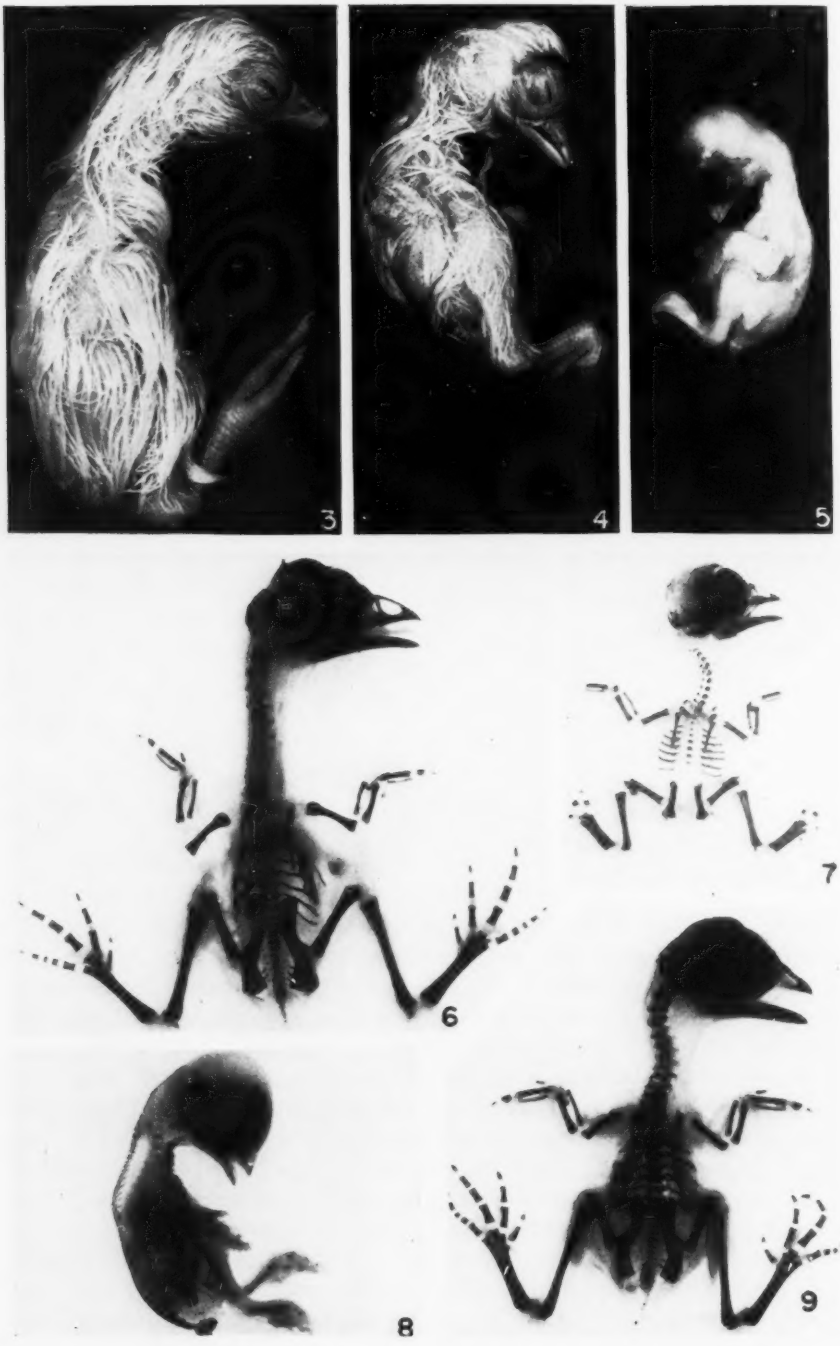


FIG. 2. The effects of cortisone acetate on the weight of chick embryos. Cortisone acetate, 2 mg./egg, was given to one group on the 4th day and another on the 8th day of incubation. Embryos were sacrified on the 8th day and at various periods thereafter.

of development with 2 mg./egg of cortisone. They were not observed in embryos treated after the 5th day of development.

The extremities did not show any outwardly apparent changes. However, in 12-day embryos stained with methylene blue for cartilage at 12 days, the humerus and the femur were found to be frequently disproportionately shortened, typically bent and carried a median protuberance (Fig. 17). These defects occurred usually in embryos which also showed beak malformations. Their incidence was not established.

The ventral body wall was markedly affected in a large proportion of the treated embryos. The most readily noticeable abnormality was a defective closure of the body wall with the resulting exterioration of the viscera. The incidence of this malformation is shown in Table 2 and Figure 1. This defect occurred in both syndromes. The results presented in Table 2 refer to material examined on the 14th day of incubation; however, the defect per-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sisted in later development and was found in 18-day embryos (Figs. 4, 5). There was also a remarkable interference with the development of the sternum, presumably due to failure of closure of the ventral body wall. These changes will be discussed in more detail in connection with syndrome II.

Syndrome II (Embryos treated 5–8 days of development). The embryos showing this syndrome were consistently dwarfed more severely than those with syndrome I. Growth retardation was directly related to the severity of the other morphological disturbances, and the various external body regions were proportionately affected with the exception of the eyes (Fig. 4). The morphological defects typical of syndrome II were found in the ventral body wall, the eyes, the integument and its derivatives, the beak and the skeleton.

Ventral body wall. As noted in connection with syndrome I, the most conspicuous abnormality affecting this part of the body was the defective closure, which resulted in an exterioration of the viscera. Depending upon the age of the embryo when cortisone was administered, closure was either partially or almost completely prevented.

During the 7th and the 8th day of normal development, the ventral body wall closes in an anterio-posterior direction and in most embryos it is found closed by the end of the 8th day, except for the umbilical area. In embryos treated on the 8th day of incubation, closure took place in the

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3. Normal 18-day chick embryo.
4. Eighteen day chick embryo, treated at 4 days with 2 mg. cortisone acetate applied to the allantoic vesicle. The embryo is moderately stunted. The head shows a facial coloboma, visible grossly as a defect in the upper beak. Feather development is within normal limits. The viscera are exteriorized.
5. Eighteen day chick embryo, treated at 8 days with 2 mg. cortisone acetate applied to the CAM. This is a stage IV "cortisone effect." The embryo is severely stunted; there is marked inhibition of feather development; the eyes are disproportionately large; there are no scales; the toes are shortened and the beak is small. The viscera are exteriorized.
6. Alizarin stained cleared skeleton of normal 18-day chick embryo.
7. Alizarin stained, clearned skeleton of 18-day chick embryo, treated at 8 days with 2 mg. cortisone acetate. Ossification is retarded; the membranous bones of the roof of the head did not form; the posterior part of the vertebral column did not ossify and the anterior part is only partially ossified, and the distal phalanges are missing.
8. Cleared skeleton of 18-day chick embryo, treated with 2 mg. cortisone acetate at 8 days of incubation. The feathers are very small and their distribution typical of stage IV "cortisone effect" (Fig. 5).
9. Alizarin stained, cleared skeleton of an 18-day chick embryo, treated at 4 days with 2 mg. cortisone acetate applied to allantoic vesicle. There is a facial coloboma and a defect of the palate; the femur is relatively shortened as compared to the tibia; but otherwise the skeleton appears to be within normal limits.

thoracic region while most of the abdominal wall remained open and the abdominal viscera were exteriorized. When cortisone was administered to 6- or 7-day embryos the closure of the ventral body wall was entirely prevented: from the coracoid area down, the visceral cavity remained open and the thoracic and abdominal viscera developed outside.

The incidence of exteriorized viscera caused by a single dose of cortisone, applied between 3 and 8 days of incubation is summarized in Figure 1 and Table 2. The maximal incidence of this defect (81% of the surviving embryos, examined on the 14th day) and its severest forms occurred in embryos treated at 6 days of incubation. Defective closure of the ventral body wall and exteriorized viscera were characteristic of both syndromes and a high incidence of these malformations occurred at the period during which the two syndromes overlapped. However, as the extremes of incidence and severity occurred in embryos treated at 6–7 days of development, these defects should be considered as more characteristic of syndrome II.

Eyes. In contrast with the general dwarfing of cortisone treated embryos, the eyeballs continued to grow. Thus, in 14- to 18-day cortisone treated embryos the eyes were disproportionately large, bulging grotesquely from the sides of the head (Fig. 5). In many of the severely affected embryos the eyelids and the nictitating membrane failed to develop, partially or entirely. The most complete failure of eyelid development occurred in embryos which received cortisone at 6 days of incubation. Treatment in later stages resulted in lesser inhibition.

According to Hamburger and Hamilton (9) the eyelids in the chick embryo begin to develop on the 7th day as folds of the integument around the eyes. The effect of cortisone seemed correlated with the degree of eyelid development at the time of treatment. Thus, in embryos treated on the 6th day, the formation of the initial folds was completely inhibited. In 8-day embryos eyelid development was stopped by cortisone at the stage reached at the time of treatment.

The integument and its derivatives. The skin of freshly sacrificed cortisone treated embryos had a pale, yellowish appearance and it was frequently severely edematous. The most conspicuous changes in the integument of such embryos were inhibition and aberrations of the development of feathers, scales and claws.

The inhibition of feather development in embryos showing syndrome II ranged from a complete absence of plumage to the presence of retarded and aberrant feathers in certain areas of the skin (Figs. 5, 8). There was a correlation between the severity of feather inhibition and the age of the embryo at the time of treatment with cortisone. This correlation was clearly evident when the dorsal feather tracts of embryos treated at 6, 7 and 8 days of development were compared on the 14th day of incubation. Most of the affected survivors treated at 6 days of development with 1.5 mg./egg of

cortisone showed a complete absence of feathers on the back. In the remainder there was some development of scattered feathers in this area. Embryos treated at 7 days had 2–7 rows of small and deformed feathers on each side of the mid-dorsal line. Their pattern of distribution resembled closely that of the dorsal feather primordia in the normal 7-day embryo (stage 30–31) (9). In embryos treated at 8 days, a greater number of dorsal feather tracts was present and the development of the individual feathers was more advanced, though of aberrant structure. Their general pattern of distribution conformed closely with that of the 8-day normal embryo (stages 32–33) (9).

In normal embryogenesis, dermal thickenings, representing the beginning formation of feather primordia, appear on the mid-dorsal line of the embryo on the 6th day of development (15–18). Similar thickenings appear lateral to the initial ones in a sequential manner, following a typical pattern. Due to this successive appearance of new feather primordia there are present simultaneously in this, as well as in other feather forming areas, primordia in various stages of development. The formation and development of the early dermal thickenings was found to be completely inhibited by cortisone. As only such early structures are present in the 6-day embryo, treatment at this age completely eliminated feather formation. In embryos treated later in development, those primordia that, at the time of treatment had already emerged above the level of the skin, proceeded to develop but in a markedly stunted and abnormal manner. Thus, the eventual patterns of plumage in the treated embryos reflected the various degrees of development and susceptibility of the primordia at the time of treatment (Figs. 21, 22).

Scales and claws were entirely absent in all the severely affected embryos. These structures begin to develop in the embryo at about the 11th day and, like the feathers, derive of mesodermal and ectodermal components. Cortisone administered between the 6th and 8th day of development prevented, in many instances, the formation of primordia of these structures.

The skeleton. Cortisone affected strikingly the ossification of various endochondral and membranous bones, retarding the process in some and completely preventing it in others. Complete inhibition of formation of certain skeletal constituents was also prevented.

The skull was rounder in outline than in normal embryos, showing a pronounced curvature in the occipital area. The beak was frequently shortened, the upper part being occasionally shorter than the lower and bent. However, all of its skeletal constituents were present in their appropriate positions, in marked contrast with the beak defects characteristic of syndrome I. On the other hand, there was a conspicuous developmental failure in the roof of the skull: as clearly seen in alizarin-stained embryos, there was no ossification of the membranous bone rudiments in

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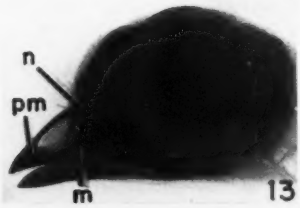

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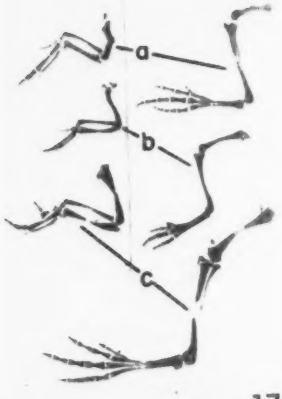

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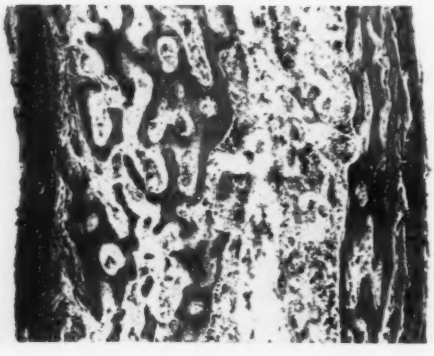

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this area. There was, thus, a distinct difference between the response to cortisone of the membranous bones in the beak region and that of the osteogenic membranes in the roof of the skull.

The cartilaginous primordia of the vertebral column developed in an orderly manner, even in the most severely affected embryos. Their ossification was retarded to various degrees in direct relation to the severity of the cortisone syndrome (Fig. 7). Ossification of vertebrae in normal embryogenesis takes place in an anterior-posterior order, so that a series of stages exists in a single embryo. This differential along the vertebral axis is reflected in the manner of response to cortisone. Figure 7 shows an 18-day embryo treated with cortisone at 8 days; it is stained with alizarin for bone. There is a sequential reduction in the degree of stainability of the vertebrae: the cervical vertebrae show partial ossification of the centra and

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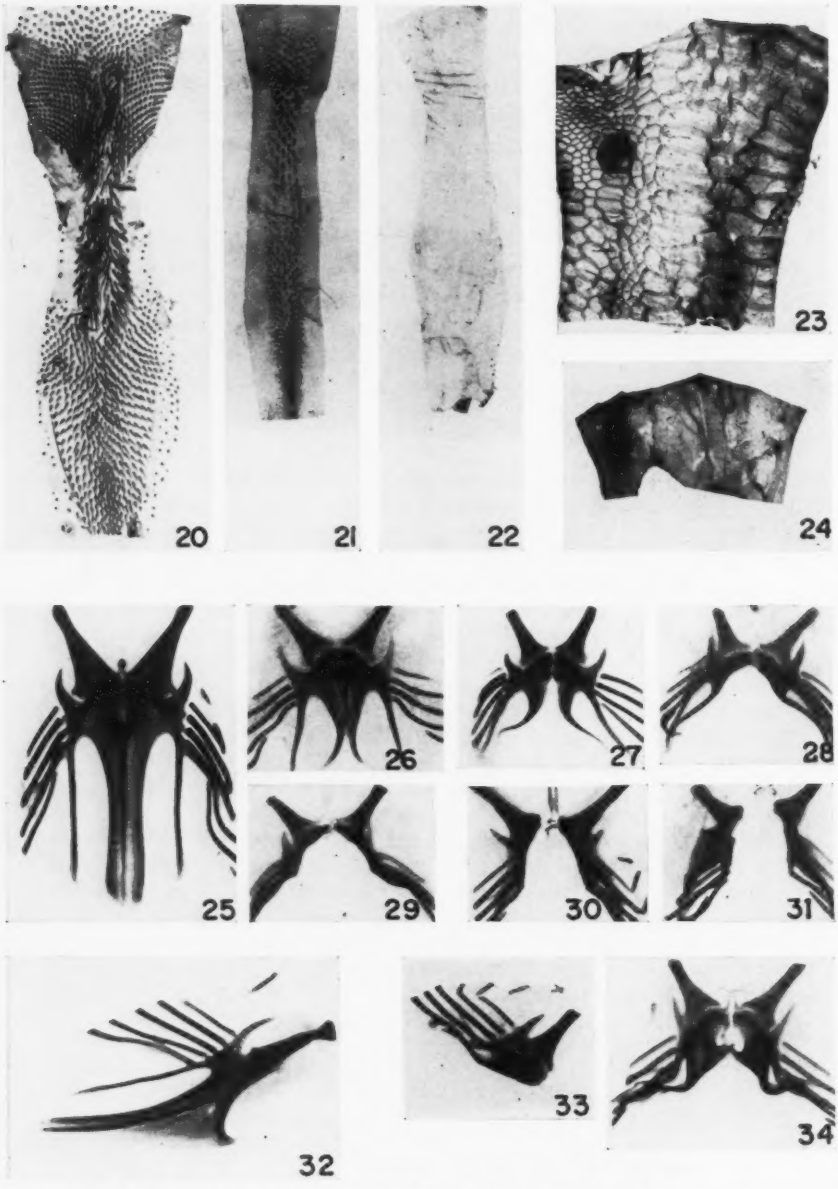
10. Alizarin stained, cleared specimen of base of brain and upper beak of normal 18-day embryo. (*pm* is the premaxilla, *m* the maxilla, and *pa* the palate.)
11. Similarly prepared specimen of the upper beak and base of brain of an embryo treated at 4 days with 2 mg. cortisone acetate and sacrificed at 18 days. The small premaxillae (pm) are seen; the maxillary bones (m) are small and deformed and dislocated, so that a bilateral gap exists between the premaxillae and maxillae. The palatal bones (pa) are shortened, flattened and directed laterally to join the defective maxillae.
12. Similarly prepared preparation of an embryo treated at 8 days with 2 mg. cortisone acetate and sacrificed at 18 days. Ossification is light but all bones are present in their proper position (compare with Figs. 10, 11).
13. Similarly prepared preparation showing a profile of the normal head of an 18-day embryo. (*n* is the nasal bone.)
14. Similarly prepared profile of 18-day embryo, treated with 2 mg. cortisone on 4th day. The nasal bone (n) is curved posteriorly. The coloboma is due to the absence of the anterior process of the maxilla (m), and the posterior dislocation of the remaining portion of the maxilla (m), together with the deficient premaxilla (pm).
15. Methylene blue stained, cleared skeleton of a normal 18-day chick embryo.
16. Methylene blue stained, cleared skeleton of an 18-day chick embryo treated with 2 mg. cortisone acetate at 8 days. Due to retarded ossification, the entire shaft of the long bones take the cartilage stain. (Compare with Fig. 15.)
17. Wings and legs of 12-day embryos cleared and strained with methylene blue to demonstrate cartilage (wings on left, legs on right).
    (a) Treated at 4 days with 2 mg. cortisone acetate. The femur and humerus are short and bent and there is a protuberance in the midshaft of the humerus and femur, presumbably due to the sharp angulation.
    (b) Treated at 8 days with 2 mg. cortisone acetate. The long bones are shortened and the distal phalanges are missing.
    (c) Normal control.
18. A section through the shaft of the femur from a normal 16-day old embryo decalcified and stained with hematoxylin and Biebrich scarlet.
19. A section through the shaft of the femur from a 16-day old embryo treated with cortisone at 8 days of incubation. The shaft is filled with cartilage which did not undergo ossification, but there is some perichondral bone formation.



20. Whole mount of skin from the back of an 11-day old embryo showing the normal distribution of the feather tracts. (Stained with hematoxylin and cleared.)
21. Whole mount of skin from the back of a 14-day old embryo, treated with 2 mg. cortisone acetate at 7 days. Although this is a 14-day embryo, the existing feathers are small and abnormal, and only a few feather tracts developed. (Compare with 11-day embryo, Fig. 20.)

of the neural arches. There is even less calcified tissue in the thoracic vertebrae of which only the centers show ossification. From there on there is no demonstrable bone. Figure 7 should be compared with Figure 16 which is of a similar embryo, stained with methylene blue for cartilage and shows the completeness of the cartilaginous elements of the vertebral column.

The extremities of the affected embryos also showed various anatomical and histological defects. The distal phalanges were frequently missing. The course of ossification in the long bones differed markedly from the normal. The endochondral ossification of the proximal skeletal elements of the appendages was very retarded. Histological examination of this material showed that the shaft consisted of a continuous core of cartilage surrounded by perichondral bone. There was no typical bone marrow cavity (Fig. 19).

The sternum. The sternum underwent striking changes in all embryos with defective closure of the body wall, their extent corresponding to the severity of the ventral body wall malformation. These changes ranged from a partial failure of the sternal plates to unite in their posterior region, to a complete absence of closure along the whole length of the sternum, with consequent aberrations in the structure of the separate sternal elements and the positioning of the ribs. The description of these changes will be facilitated by a brief account of the normal development of the avian sternum, described in detail by Fell (10).

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22. Whole mount of the skin from the back of a 14-day old embryo, treated with 2 mg. cortisone acetate at 6 days. No feathers are present.
23. Whole mount of skin from the leg of a normal 16-day embryo showing the scales.
24. Whole mount of skin from the leg of a 16-day old embryo treated with 2 mg. cortisone acetate at 8 days. The scales are not present.
25. Methylene blue stained, cleared sternum of a normal 12-day embryo (ventral view).
26. Ventral view of the sternum of a 12-day old embryo, treated with 2 mg. of cortisone acetate on the 8th day of incubation (presumbably late 8th day). The posterior parts of the sternal plates are not fused in the mid-ventral line.
27. Ventral view of sternum from a 12-day old embryo treated with cortisone acetate on the 8th day (presumably earlier than Fig. 26).
28, 29. Ventral view of sternum from a 12-day old embryo treated with cortisone on the 7th day. Fig. 28 hows only fusion of the anterior portion of the sternal plate, although the sternal plates have moved medially. In Fig. 29 the sternal plates remained laterally placed.
30, 31. Ventral view of sternal plates from 12-day old embryos treated with 2 mg. cortisone at 6 days of incubation. Sternal plates are not closed and they are laterally placed.
32. Lateral view of Fig. 25, normal 12-day embryo.
33. Lateral view, Fig. 30.
34. Ventral view of sternal plates from 12-day embryo treated with 2 mg. of cortisone on the 4th day of incubation. The sternal plate is partially fused anteriorly.

The sternum starts its development as a pair of primordia which fuse later. They arise on the 6th day of development as mesenchymal plates in the dorso-lateral wall of the thorax. Between the 7th and 8th day the plates move to a vertical position on both sides of the thoracic cavity. Concurrently, four rapidly expanding cartilaginous centers appear in each plate. On the 8th day the anterior edges of the plates begin to join at the ventral midline. The closure of the sternal plates continues in an anterior-posterior direction and is completed by the 11th day of development.

In embryos treated on the 8th day of development closure of the sternal plates occurred only at their anterior ends (Fig. 26). When cortisone was given on the 6th day, the sternal plates remained in a vertical position on both sides of the thoracic cavity. Moreover, as the separate plates continued to grow in this abnormal position, they became distorted in shape (Figs. 30, 31).

Comparing the above aberrations with the normal pattern of development, it seems that cortisone did not prevent the development of the sternal plates or their movements. In embryos treated on the 6th day, the mesenchymal primordia developed into cartilaginous plates which resembled, essentially, the normal structures, and moved from their primary place in the dorso-lateral wall to a vertical position. Only the final steps which depend on the normal closure of the ventral body wall were affected.

DISCUSSION

A detailed description of some of the effects of cortisone, administered at various stages in the development of the chick embryo, has been presented. Depending on the stage of development at the time of cortisone treatment, different patterns of response have been observed. Each system in the embryo appeared to reach a stage of maximum and specific responsiveness to cortisone at different periods of development, so that a wide spectrum of embryonic disturbances was found. For the purposes of analysis, however, the diverse effects have been grouped into two distinctive syndromes. Syndrome I is representative of the embryos treated at 3–5 days, and syndrome II of the embryos treated at 8 days of incubation (Table 3). The correlation between the developmental stage of an embryonic region and the patterns of response to treatment is essential for the interpretation of the results of these experiments.

Not only is the time of cortisone administration important, but so is the route (2). Single doses of cortisone given by yolk sac injection at 4 and 8 days were about one-third as active by weight as when the drug was inoculated onto the CAM at 8 days. It is postulated that cortisone is rapidly absorbed from the CAM, acts promptly on the embryos, and any unused portion is catabolized or stored in various compartments of the egg, where as cortisone injected into the yolk sac is more slowly absorbed, and much larger doses are necessary to produce an effect. In this study the CAM

TABLE 3. DEVELOPMENTAL DEFECTS IN THE CHICK EMBRYO AFTER APPLICATION OF CORTISONE ACETATE (2 MG./EGG) AT 4 AND 8 DAYS OF INCUBATION, AS OBSERVED ON THE 16TH DAY OF INCUBATION

	Syndrome I treated at 4 days	Syndrome II treated at 8 days
Weight inhibition	Average weight 6 gm.	Average weight 4 gm.
Feathers	Normal	Feather formation limited to the tracts present at the time of injection; subsequent feather tract formation was inhibited
Scales	Normal	Formation of scales inhibited
Skeletal defects:		
(a) Endochondral bones	Shortening and bending of femur and humerus (Fig. 17a). Ossification as observed on 16th day normal	Distal phalanges missing. Vertebral ossification retarded. Endochondral ossification of long bones retarded
(b) Membranous bones	Facial skeleton: facial coloboma with cleft palate (occasional slight shortening of the lower beak). Bones of the head roof normally ossified	Facial skeleton: the beak shortened. Upper beak bent. Bones of the head roof not ossified
Exteriorized viscera	Low incidence	Highest incidence after treatment at 6 and 7 days. Lower after treatment at 8 days

route was employed to give a rapid uptake of cortisone, and thus produce sharp effects on the embryo in relation to the time of treatment.

Whether the morphological alterations produced by cortisone in the embryo are due to its direct effect on the affected structures, or to indirect effects is not known. A direct effect seems to be a reasonable interpretation of the defects typical of syndrome I. Cleft palate and facial coloboma occur only when cortisone is administered during the short period when the palate and jaws are being laid down; that is, while movements of cells and of cell groupings, involved in the building of these structures, are in progress. At a later period, when the scaffolding of these structures has been established, cortisone no longer interferes with their development. At a similar sensitive stage facial coloboma and cleft palates have been produced by other agents (11); these include azaserine (12) and boric acid (13). Whether these chemically unrelated agents affect the same basic processes in the sensitive tissues requires further analysis.

A direct effect of cortisone on morphogenetic processes also seems likely in the case of feather development. It has been shown in a number of studies (15–22) that there is a mutual morphogenetic dependence between the dermal papilla and the overlying ectoderm, both in the early and in the further development of the feather primordium. Any interference with the functional integrity of these components or their interaction might be, therefore, expected to inhibit or modify development in various degrees.

The observations on the effects of cortisone on the feather birds suggest

these conclusions:

(a) Cortisone prevents the formation of new feather papillae.

(b) Papillae affected before the ectodermal response has set in do not give rise to feather buds.

(c) Young feather buds affected by cortisone continue to develop and grow but in a stunted and abnormal manner.

The available data do not indicate whether cortisone acts solely on the dermal papillae or also directly affects the overlying ectodermal cells; both of these tissues may be targets. The possibility also exists that cortisone may lead to an impairment in the reciprocal interaction between the two tissues.

A striking example of a type of indirect effect of cortisone on organ development is provided by the fate of the sternum in treated embryos. Our observations have shown that the disturbance in sternal development is due to partial or complete failure of the body wall to close, and thus to exert the mechanical influence necessary to bring the sternal plates together. The mechanism whereby cortisone inhibits body wall closure has not been determined.

Cortisone may affect differently histogenetic processes of presumably comparable type. For instance, while calcification of the membranous bones of the jaws is not noticeably impaired, that of the membranous bones of the skull roof is markedly retarded. This is not surprising in view of the fact that tissues of apparently similar histogenetic appearance do not necessarily proceed along identical lines of development, and do not reflect identical processes of cellular arrangement and reaction (14).

Pickman *et al.* (23) recently found that cortisone inhibits embryonic growth when multiple doses are applied to the embryonic membranes during early development. Our data clearly indicate that certain structures in the early embryo are differentially susceptible to the action of cortisone. It has been suggested (2) that the most severe growth-inhibiting effect of cortisone occurs on the 8th day of incubation and thereafter. This could be due in part to a direct effect of cortisone on the susceptible tissues, but indirect effects through the developing endocrine system and interference with nutrition due to disturbances in the development of embryonic membranes may be implicated (2).

It is apparent that the embryo, in the course of its development, shows striking regional differences in its susceptibility to cortisone. A satisfactory interpretation of these diverse effects and the underlying metabolic mechanisms must await a clearer understanding of cortisone action. Cortisone suggests itself as a useful tool for the analysis of developmental mechanisms. The chick embryo seems to be an excellent system for studying the possible localization and the function of cortisone and related adrenal steroids, since susceptible cells appear and disappear in such a regular

sequence and their responses give rise to striking, readily identifiable developmental disturbances.

REFERENCES

1. Landauer, W.: *Endocrinology* **41**: 489. 1947.
2. Karnofsky, D. A., L. P. Ridgway and P. A. Patterson.: *Endocrinology* **48**: 596. 1951.
3. Karnofsky, D. A.: *Trans. N. Y. Acad. Sci.*, Series II **13**: 61. 1950.
4. Stock, C. C., D. A. Karnofsky and K. Sugiura: in "Symposium on Steroids in Experimental and Clinical Practice." p. 50, Blakiston Co., Phila., 1951.
5. Kilbourne, E. D. and F. L. Horsfall, Jr.: *Proc. Soc. Exper. Biol. & Med.* **76**: 116. 1951.
6. Cavallero, C., A. Di Marco, L. Foon and G. Sala: *Proc. Soc. Exper. Biol. & Med.* **81**: 619. 1952.
7. Evans, H. J.: *Proc. Exper. Biol. & Med.* **83**: 31. 1952.
8. Zwilling, E.: *J. Exp. Zool.* **199**: 197, 1948.
9. Hamburger, V. and H. L. Hamilton: *J. Morph.* **88**: 49. 1952.
10. Fell, H. B.: *Phil. Trans. B* **229**: 407. 1939.
11. Karnofsky, D. A.: *Stanford Med. Bull.* **13**: 247. 1955.
12. Dagg, C. P. and D. A. Karnofsky: *J. Exp. Zool.* **130**: 555. 1955.
13. Landauer, W.: *J. Exp. Zool.* **120**: 469. 1952.
14. Weiss, P. and A. Moscona: *J. Embryol. & Exper. Morph.* **6**: 238. 1958.
15. Lillie, F. R. and H. Wang: *Physiol. Zool.* **14**: 103. 1941.
16. Lillie, F. R. and H. Wang: *Physiol. Zool.* **16**: 1. 1943.
17. Wang, H.: *Physiol. Zool.* **16**: 325. 1943.
18. Watterson, R. L.: *Physiol. Zool.* **15**: 234. 1942.
19. Saunders, J. W.: *Physiol. Zool.* **16**: 325. 1947.
20. Goff, R. A.: *J. Morph.* **85**: 443. 1949.
21. Cairns, J. M.: *Anat. Rec.* **111**: 452 (abst.). 1951.
22. Cairns, J. M. and J. W. Saunders: *J. Exp. Zool.* **127**: 221. 1954.
23. Pickman, D. S., A. Ridley, M. Orgel and H. T. Blumenthal: *Endocrinology* **64**: 790. 1959.

11β-HYDROXY DEHYDROGENASE SYSTEM ACTIVITY IN THYMI OF MICE FOLLOWING PROLONGED CORTISOL TREATMENT

THOMAS F. DOUGHERTY, MARTHA L. BERLINER AND DAVID L. BERLINER[1]

Department of Anatomy, University of Utah, College of Medicine, Salt Lake City, Utah

ABSTRACT

The purpose of these studies was to compare the amount of transformation of cortisol to cortisone produced by lymphocytes of untreated animals and those of animals treated with large doses of cortisol for a period of several days. The lymphatic organs (spleens, lymph nodes and thymi) of 50 mice of the CBA strain which were given cortisol acetate (1 mg.) daily for 9 days were compared to untreated CBA mice of the same age and sex for the capacities of these cells to transform cortisol to cortisone. The amounts of differences of 11β-hydroxydehydrogenase system activity present in the two groups of animals were estimated by incubating equivalent amounts of tissue (300 mg.) from each of the two groups with cortisol-4C^{14} (11.5 mμM). In untreated animals the abilities of the tissues to make the transformation were highest in lymph nodes, second highest in spleen and very low in the thymus. Following prolonged treatment with large doses of cortisol it was found that the thymus increased its capacity to transform cortisol to cortisone by 217%. Lymph nodes showed a small but definite increase and there was a slight decrease in the spleen. The factors which might be brought into play to explain this enormous increase in thymic activity are discussed in the body of the paper and the presence of 11β-hydroxydehydrogenase in various cell types (normal immature, cortisol-treated lymphocytes and malignant lymphocytes) are discussed.

AMONG the endogenously formed adreno-cortical hormones which produce karyorrhexis of lymphocytes, cortisol was found to be the most active (1). Immature or reticular lymphocytes were found to be more resistant to this particular action of cortisol (1). In other experiments it was observed that malignant lymphocytes have great ability to transform cortisol to metabolites which are devoid of the capability to produce lymphocyte destruction (2, 3). Thus, with respect to the lymphocyte destructive effect of cortisol, these substances are ineffective. Cortisol in small amounts which probably approaches physiological concentration, produces karyorrhexis of normal but not malignant lymphocytes (4). Similarly, mature lymphocytes are destroyed by concentrations of cortisol which do not affect immature lymphocytes (5, 6).

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The purpose of this investigation is to ascertain whether lymphocytes taken from animals treated daily for 9 days with large doses of cortisol have developed greater abilities to metabolize this hormone to ineffective transformation products. Since smaller mature lymphocytes are most susceptible to cortisol effects, successive treatment with greater than physiological amounts of cortisol destroys small cells and thus leaves the immature reticular lymphocytes (6, 7). Accordingly, by incubating cortisol-4C^{14} with these cells one can compare both quantitatively and qualitatively the capacities of the differentiated and undifferentiated cells to catabolize cortisol.

MATERIALS AND METHODS

Two groups of female CBA mice weighing 18 to 25 gm. were used. There were 50 mice in each group. All animals were maintained on Purina Dog Chow and water *ad lib.* with protein and vitamin supplements. Animals of group 1 were treated daily intraperitoneally with 1 mg. cortisol acetate each, for 9 consecutive days. Group 2 animals were used as untreated controls. Two hours after the last cortisol injections, both control and experimental animals were sacrificed by spinal severance. Thymi, lymph nodes and spleens were collected from both groups. Samples of the lymphatic organs were taken for histological observation. The collected thymi, lymph nodes or spleens of each group were weighed and minced. Three hundred mg. samples of each organ mince were placed in incubation flasks (125 ml. Erlenmeyers) containing 11.5 mμM (4.2 μg.) of chromatographically pure Cortisol-4C^{14} (Specific Activity: 3230 cpm/μg.) in 10 ml. phosphate buffer at pH 7.4. Incubation time was 3 hrs at 37° C. The mixture was shaken gently during this time. Other flasks containing identically minced tissues as those described above were prepared for extraction without incubation (zero controls). The minced material was then extracted with the acetone-chloroform technique previously described (8) for the preparation of an extract suitable for paper chromatography. The chromatographic techniques were those of Zaffaroni (9). For determination of specific activities (11) radioactivity was quantitated directly on the paper chromatogram by employing area analysis of the radioactive peaks and the β-ray self-absorption factor, which in our case is 6.12 (12). Therefore:

$$R = A \times 6.12$$

where R is equal to radioactivity in counts/min. and A is equal to the area in sq. cm.

The several radioactive areas of the chromatogram were eluted separately and identified by rechromatography in the presence of nonradioactive index compounds (10).

Quantitation of the index steroids with the $\alpha\beta$-unsaturated ketone which absorbs at 240 mμ maximum was performed in a model DU Beckman spectrophotometer using silica cells of 1-cm. light path containing either 3 ml. or 0.5 ml. of the compound dissolved in methanol. The following formula was applied:

$$\mu g. = \frac{F \times 240\ m\mu_{max}}{1000}$$

where F is equal to the extinction coefficient of the particular steroid as described by Cruz and Gomez-Mont (13). Specific activity was determined by area analysis, followed by elution and steroid quantitation of the radioactive region.

When a derivative was prepared, the foregoing procedure was repeated with the exception that a correction factor was employed for the change in molecular weight:

$$\mu g._r = \mu g._d(K)$$

where $\mu g._r$ is equal to equivalent micrograms, and $\mu g._d$ is equal to the weight of the derivative.

$$K = \frac{\text{molecular weight of the parent steroid}}{\text{molecular weight of the derivative}}$$

Therefore, the relative specific activity of the derivative was calculated according to the following formula:

$$\text{Relative S.A. (counts/min/}\mu g._r) = \frac{A \times 6.12 \times 1000}{F \times 240\ m\mu_{max}(K)}$$

where A is equal to the area under the radioactive peak of the derivative.

$$\frac{F \times 240\ m\mu_{max}}{1000}$$

is equal to the weight of the derivative in micrograms.

RESULTS

Identification of compounds

The major compound other than cortisol, isolated and identified from the incubated control and experimental animals, was cortisone. From the zero controls only cortisol was isolated. The identification of cortisone was done by using the following procedure: A mixture of the radioactive unknown (3400 cpm) plus authentic cortisone (64 μg.) migrated at the same rate (R_f 0.65, Specific Activity 52.0 cpm/μg.) in the chloroform-formamide system (authentic cortisol exhibits an R_f of 0.3 in the same system). One half of this mixture, when subjected to a strong oxidation with CrO_3 (10) was converted to 4-androstene-3,11,17-trione. This oxidation product had an R_f of 0.50 and a specific activity of 52.8 cpm/μg. in benzene-formamide, and exhibited the same chemical and physical properties as 4-androstene-3, 11,17-trione. The other half was acetylated (10), showing a specific activity of 53.0 cpm/μg. upon benzene-formamide chromatography, and had the same chemical properties as cortisone-21-acetate (R_f 0.25).

The mμM of steroid converted per 300 mg. of tissue in 3 hrs is shown in Table 1. In untreated controls it may be seen that the lymph nodes have the highest capacity to dehydrogenate the eleven position of cortisol. The spleen is next most active and thymus has least activity. Conversely, this is not the case following cortisol treatment of the animals. From data obtained it was obvious that the thymi from cortisol treated mice showed the highest level of transformation of cortisol to cortisone (Fig. 1 and Table 1). Second highest activity was exhibited by lymph nodes. Following cortisol pre-treatment, a decrease in metabolic activity was exhibited by splenic tissue (Table 1).

Histological observations

There is a lack of demarcation between cortical and medullary areas of lymph nodes of cortisol treated animals. Lymphocytes have pycnotic nu-

TABLE 1. TRANSFORMATION OF CORTISOL-4C^{14} TO CORTISONE-4C^{14} BY LYMPHATIC TISSUES *in vitro*. THE % CONVERSION IS GIVEN FOR THE CONTROL GROUP AND FOR TISSUES OF ANIMALS TREATED WITH 1 MG OF NONRADIOACTIVE CORTISOL PER DAY FOR 9 DAYS. EACH INCUBATION FLASK CONTAINED 300 MG OF TISSUE AND WAS INCUBATED FOR 3 HOURS AT 37° C

Tissue	mμmoles Cortisol incubated	mμmoles Cortisone isolated	Average % conversion
Thymus			
Control	11.5	1.2 (0.9–1.4)*	10.4
Cortisol treated	11.5	3.8 (3.4–4.2)	33.0
Spleen			
Control	11.5	2.0 (1.9–2.2)	17.3
Cortisol treated	11.5	1.6 (1.5–1.8)	13.9
Lymph Node			
Control	11.5	2.4 (2.2–2.6)	20.8
Cortisol treated	11.5	2.9 (2.6–3.1)	25.2

* Minimum-maximum of four (4) incubations.

clei and there is a profound reduction in the amount of cytoplasm. Macrophages and reticular lymphocytes are apparently decreased as compared to those of non-treated animals but are proportionately far greater in number than the mature lymphocytes.

Chronic cortisol administration also produced a marked decrease in size and weight of the spleens. Sections of spleen show a partial disappearance of demarcation between white and red pulp. Pyknosis of white pulp lymphocyte nuclei was evident and immature cells appeared to be resistant to nuclear and cytoplasmic destruction. The red pulp contained many reticular cells. Areas of myelo- and erythropoiesis were frequently observed.

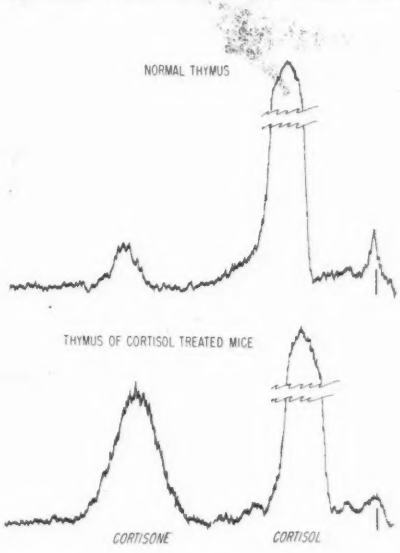

FIG. 1. Radioactive tracings of paper chromatographic zones of incubations of thymi of experimental and control mice in the chloroform-formamide system. Both chromatograms were counted at 1000 cpm full scale on an automatic strip counter. Quantitation was performed by area analysis of the peaks (see Ref. 12). The R_f of cortisol is 0.3 and the R_f of cortisone, 0.65. Note the increased amount of radioactivity in the cortisone peak from the cortisol-treated mice.

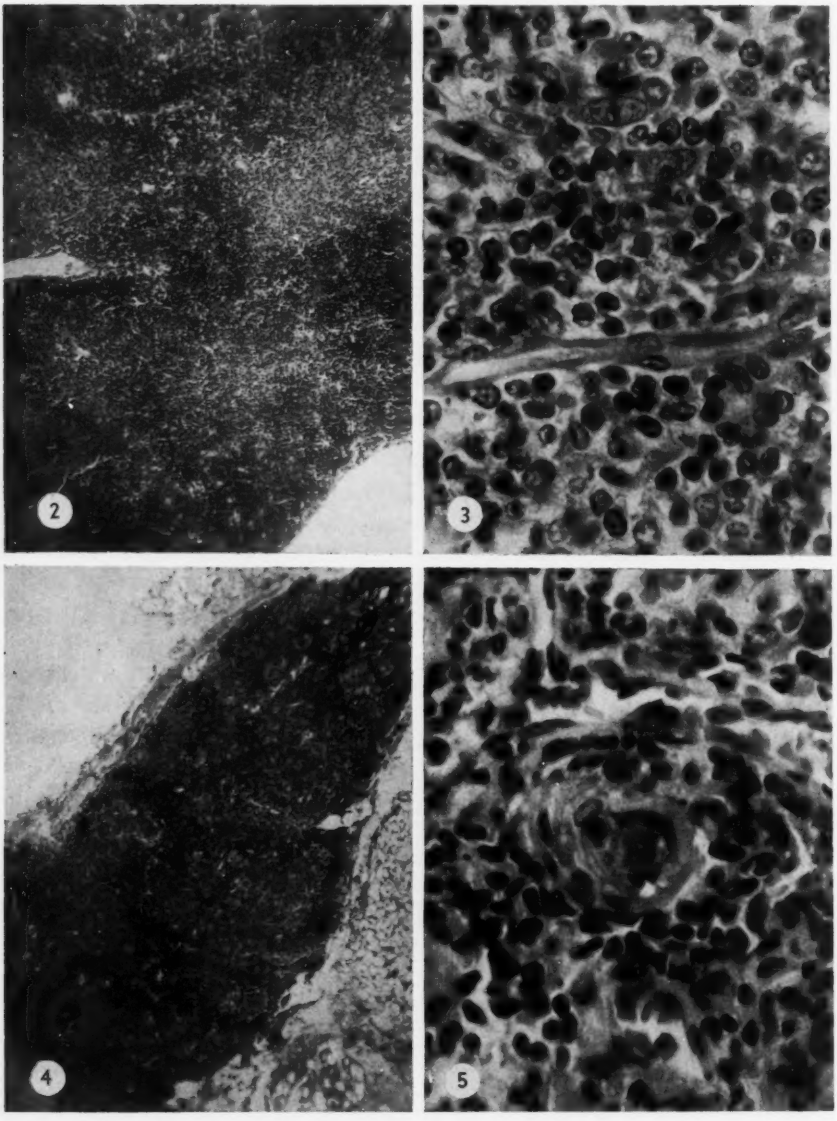

FIG. 2. Thymus of untreated CBA mouse. Note demarcation of cortical and medullary area. Staining H.E. Magnification ×92.

FIG. 3. Medullary area of untreated CBA mouse (Fig. 2). Large leptochromatic nuclei are those of entodermal reticular cells. Small dense nuceli belong to lymphocytes. Staining H.E. Magnification ×790.

FIG. 4. Thymus of chronic cortisol treated mouse (1 mg. daily for 9 days). Note reduction in cortical portion and absence of cortical-medullary demarcation. Only

Although mitotic figures were absent from the lymphatic organs, the mylopoietic cells showed numerous mitoses.

The thymi of hormone treated animals are exceedingly small. The medullary portion does not appear to be decreased as compared to control nontreated animals (Figs. 2, 4) but there is almost a complete absence of the cortical portions (Fig. 4). Pycnotic lymphocytes and some immature lymphocytes are scattered about in the endodermal reticular stroma of the medulla (Fig. 5). The endodermal reticular cells and the lymphocytes are the sole cellular types found (Fig. 5). A most interesting event was the development of Hassall's corpuscles in the cortisol treated animals (Fig. 5). The mouse and rat are two species in which Hassall's corpuscles are not found in the normal animal. Unlike guinea pigs and humans in which these bodies increase in number with increase in age, they do not even appear in old mice.

DISCUSSION

The relationship between cortisol action and its metabolism by hepatic and extrahepatic cells has been investigated extensively in our laboratory. The focus of attention has been on those cells primarily affected by cortisol in conditions in which this hormone exerts actions of physiological significance. The results of many studies indicate that fibroblasts are the main target cells of cortisol action when this substance exerts an antiphlogistic effect (14, 15). The interrelationship between cortisol and the capacity of fibroblasts to metabolize this hormone have been studied extensively *in vitro*, using fibroblasts from the connective tissue of the mouse (17), *in vitro* in tissue culture of human fibroblasts (18) and finally, for fibroblasts taken from areas of inflammation (19). The details of these investigations are given in separate publications and the implications of these studies for the antiphlogistic influence are given in reviews (16, 20, 21). In general it appears that among numerous normal cell types which have now been studied, fibroblasts are the most potent of extrahepatic cells in their capacity to catabolize cortisol (16). Unlike the liver cells, fibroblasts cannot reduce the 3 keto position. They have only slight capacity to reduce the Δ^4 double bond and they cannot conjugate this steroid (11, 16). However, they are able to perform all other oxidations and reductions of substituted groups on the cyclopentaneperhydrophenanthrene nucleus (gonane nucleus)* which have been mostly attributed to liver function (11, 22).

* The term "gonane nucleus" has been accepted at the meeting of the Ciba Foundation, London, May 30th to June 15th, 1950 (*Chemistry and Industry*, SN1–11, June 23, 1951).

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medullary portion remains and is shown in detail in Fig. 5. Staining H.E. Magnification ×92.

FIG. 5. Same as Fig. 4. Entodermal reticular cells and pycnotic lymphocytes remain. Note Hassall's corpuscle in center of the field. Staining H.E. Magnification ×790.

From the amount of catabolism it seems clear that this connective tissue cell which sequesters cortisol (14), largely controls the rate of cortisol inactivation. None of the conversion products have anti-inflammatory activity (23). It is apparent that unmodified cortisol exerts an anti-inflammatory effect at the site of inflammation and that it does so by increasing fibroblastic resistance to destruction and thereby reduces cytotoxin elaboration (24).

In addition to the fact that peripheral cortisol metabolism influences the amount of available active hormone, it is of some interest to inquire whether steroid hormone conversions play a fundamental chemical role in the antiphlogistic effect or whether they are primarily related to a turnover of an active form, so that rate of catabolism regulates the amount of available cortisol at the cellular level. It is true, of course, that these actions are not mutually exclusive and steroid conversions are essential to satisfy hormone concentration via pituitary adrenal mediation (25) and also may be related to hormone action (26).

Another target cell for cortisol action is the lymphocyte. The degree of influence of cortisol on the destruction and inhibition of growth of this cell type is among the most dramatic effects of any hormones on any cellular activities (27). It is interesting, then, in contrast to fibroblasts that mature lymphocytes have little, if any, capability of catabolizing cortisol in spite of the fact that they so delicately react to degrees of concentration of hormone acting upon them (2, 3). Here, then, appears to be at least one site and one type of hormone action in which unmodified cortisol acts as such and its catabolism is comparatively negligible.

Although normal mature lymphocytes are unable to catabolize cortisol to cortisone to any extent, it is evident that after prolonged hormone treatment, the thymic and lymph node cells which survive can increase this transformation. The thymic cells have developed an enormous amount of 11β-hydroxydehydrogenase activity (Fig. 1). At first glance it would appear that the lymphatic organ had developed an adaptive enzyme; however, it should be kept in mind that at the end of the treatment period the cell population is considerably different (Figs. 3, 5). The number of immature lymphocytes and reticuloendothelial cells per unit weight of tissue is vastly greater than in the non-treated organs. Thus, at this time it is impossible to determine whether the remaining cells have developed any greater ability to transform cortisol than non-treated immature cells. In any case, it is evident that immature lymphocytes resemble leukemic lymphocytes in that they can catabolize cortisol to cortisone. However, normal immature lymphocytes, unlike leukemic lymphocytes, apparently cannot catabolize cortisol to all products of conversion produced by leukemic cells (2, 3). This difference now appears to be both quantitative and qualitative because it is now known that lymphatic leukemic cells from different leukemic animals all transform cortisol, but to different products

and different amounts (unpublished observations). Immature lymphocytes and reticular cells possibly have in common with lymphatic leukemic lymphocytes a greater amount of 11β-hydroxydehydrogenase system. Enzymes capable of attacking other positions are not present in normal immature cells in great quantity.

The catabolic activities of fibroblasts and lymphocytes were contrasted above. Much larger amounts of cortisol are necessary for fibroblastic destruction (28) than for karyorrhexis of lymphocytes. Cortisol in comparatively large amounts inhibits fibroblastic growth of some strains of tissue culture fibroblasts (29). A strain of fibroblasts, however, which was resistant to cortisol growth inhibition catabolized 100% more cortisol per unit time than non-resistant strains (unpublished observations). Thus, the generalization appears warranted for fibroblasts, leukemic cells and immature lymphocytes that when these cells can catabolize cortisol, this hormone has less capacity to limit cellular multiplication.

The coenzymes necessary for the 11β-hydroxydehydrogenase system are DPN and TPN (22). It is known that these substances enter as cofactors in the transformation of cortisol to cortisone (22). It is quite possible that lymphoid cells remaining after cortisol treatment contain more of these coenzymes and for this reason have a greater 11β-hydroxydehydrogenase system activity, or, on the other hand, it seems possible that in the tissues there could be a difference in amounts of oxidized and reduced coenzymes, thus giving a difference in the products of conversion.

It is interesting that the greatest transforming activity takes place in the cortisol treated thymi. It seems possible that endodermally derived reticular cells could participate in this reaction. This possibility is being investigated in fetal mouse thymi in our laboratory.

The production of Hassall's corpuscles in stressed rats (30) or following cortisol treatment is surprising, since they do not tend to form in non-stressed or non-cortisol-treated mice. Further attention should be paid to the fact that thymi of mice and rats which secrete mainly corticosterone do not contain Hassall's corpuscles but guinea pigs and humans which secrete mainly cortisol have thymi which contain these structures.

It is noteworthy and at present unexplained that the splenic cells did not increase in their transforming ability following cortisol treatment. This further suggests that there may be some cellular activities in this regard, particularly for the thymus.

REFERENCES

1. Santisteban, G. A. and T. F. Dougherty: *Endocrinology*, **54**: 130. 1954.
2. Berliner, M. L., D. L. Berliner and T. F. Dougherty: *Proc. Amer. Assn. Cancer Res.* **2**: 94. 1956.
3. Dougherty, T. F.: The Leukemias: Etiology, Pathology, & Treatment, Acad. Press Inc., Ed. J. W. Rebuck, 293. 1957.
4. Dougherty, T. F.: Fundamental Problems and Techniques for the Study of the

Kinetics of Cellular Proliferation, Ed. F. Stohlman, Jr., Grune & Stratton, N.E., 264. 1959.
5. DOUGHERTY, T. F. AND A. WHITE: *Am. J. Anat.* **77:** 81. 1945.
6. DOUGHERTY, T. F.: *Recent Progress in Hormone Research* **7:** 307. 1952.
7. DOUGHERTY, T. F.: Proceedings of Conference on Lymphocytes, Ed. J. W. Rebuck (in press) 1958.
8. BERLINER, M. L., D. L. BERLINER AND T. F. DOUGHERTY: *J. Clin. Endocrinol. & Metab.* **18:** 109. 1958.
9. ZAFFARONI, A.: *Recent Progress in Hormone Research* **8:** 51. 1953.
10. BERLINER, D. L. AND H. A. SALHANICK: *Anal. Chem.* **28:** 1608. 1956.
11. BERLINER, D. L., B. I. GROSSER AND T. F. DOUGHERTY: *Arch. Biochem. & Biophys.* **77:** 81. 1958.
12. BERLINER, D. L., O. V. DOMINGUEZ AND G. WESTENSKOW: *Anal. Chem.* **29:** 797. 1957.
13. CRUZ, A. R. AND F. GOMEZ-MONT: *Rev. invest. clin.*, Organo hosp. enfermedad. nutricion Mex. **6:** 109. 1954.
14. DOUGHERTY, T. F., R. BIGLER, G. L. SCHNEEBELI AND H. A. SALHANICK: *Ann. N. Y. Acad. Sci.* **64:** 466. 1956.
15. DOUGHERTY, T. F. AND R. D. HIGGINBOTHAM: Fifth Ann. Report on Stress, Acta Press, Inc., Montreal, Ed. Selye & Heuser, 117. 1955.
16. DOUGHERTY, T. F. AND D. L. BERLINER: Connective Tissue, Thrombosis and Atherosclerosis, Ed. I. Page, Acad. Press, Inc. 143. 1959.
17. BERLINER, D. L. AND T. F. DOUGHERTY: *Proc. Soc. Exp. Biol. & Med.* **98:** 3. 1959.
18. SWEAT, M. L., B. I. GROSSER, D. L. BERLINER, E. H. SWIM, C. J. NABORS, JR. AND T. F. DOUGHERTY: *Biochim. Biophys. Acta.* **28:** 591. 1958.
19. BERLINER, D. L. AND T. F. DOUGHERTY: *Fed. Proc.* **17:** 189. 1958.
20. DOUGHERTY, T. F. AND D. L. BERLINER: Hormones and Atherosclerosis. Ed. G. Pincus, Acad. Press Inc. 103. 1958.
21. BERLINER, D. L. AND T. F. DOUGHERTY: Proc. Third International Symposium on Reticuloendothelial System (in press) 1958.
22. DORFMAN, R. I.: The Hormones, *V.* III, Ed. G. Pincus, K. V. Thiman, Acad. Press Inc. 589. 1955.
23. DOUGHERTY, T. F. AND G. S. SCHNEEBELI: *Ann. N. Y. Acad. Sci.* **61:** 328. 1955.
24. EYRING, H. AND T. F. DOUGHERTY: *Amer. Scientist* **43:** 457. 1955.
25. YATES, F. E., J. URQUHART AND A. L. HERBST: *Am. J. Phys.* **195:** 373. 1958.
26. TALALAY, P.: *Physiol. Rev.* **37:** 362. 1957.
27. DOUGHERTY, T. F.: *Physiol. Rev.* **32:** 379. 1952.
28. DOUGHERTY, T. F.: Adrenal Cortex. Transactions of the Second Conf., Josiah Macy, Jr. Foundation, New York, 88. 1950.
29. GROSSER, B. I. AND SWIM, H. E.: *Proc. Amer. Assn. Cancer Res.* **2:** 304. 1958.
30. GREGOIRE, CH.: *Arch. int. Pharmacodyn.* **67:** 446. 1942.

EFFECT OF STORAGE OF NaI^{131} SOLUTIONS UPON THE THYROID UPTAKE BY DAY-OLD CHICKS[1]

V. M. DOCTOR

Department of Physiology and Biochemistry, The University of Texas, Dental Branch, and the Department of Medicine, The University of Texas M. D. Anderson Hospital and Tumor Institute, Houston, Texas

ABSTRACT

Several microcuries of two to three week old solutions of NaI^{131} were spotted on sheets of filter paper and developed one dimensionally in an ascending technique using n-Butanol saturated with 3N NH_4OH. After 20 hours, the papers were air-dried and radioautographed. The "extraneous band," which moves slightly faster than the NaI^{131} band, was cut out, eluted with 50% alcohol and concentrated to 5 microcuries/ml.

Studies were conducted on the factors affecting the thyroid uptake of fresh NaI^{131} solution and of the extraneous band solution by day-old chicks. At all the dosage levels (0.1, 0.5, 1.0 microcurie) tested, the thyroid uptake of the extraneous band solution was significantly lower than that of fresh NaI^{131} solution. Administration of iodine (NaI^{127}) either orally in the diet or by injection, lowers the uptake of fresh NaI^{131} solution. However, the thyroid uptake of the extraneous band solution is enhanced by simultaneous injection of 2 to 5 μg. of NaI^{127}. The uptake of both NaI^{131} and the extraneous band solution is enhanced by the simultaneous administration of 1 to 10 mg. of cysteine hydrochloride.

SODIUM iodide-I^{131} (NaI^{131}) solutions upon storage are reported to show the presence of several bands when chromatographed one dimensionally in an ascending technique using several different solvent systems (1). The percentages of radioactivity in the bands other than NaI^{131} are reported to increase rapidly upon storage, and it is reported that addition of cysteine or NaI^{127} to the NaI^{131} solutions converges the extraneous bands into the NaI^{131} band (1). Studies on the uptake of the NaI^{131} and of the major extraneous band solution by thyroids in day-old chicks are reported to show that 20% of radioactivity from the injected extraneous band solution is collected in the thyroid in contrast to the 60% uptake of NaI^{131} (1). The present report is concerned with the effect of oral or parenteral iodine (NaI^{127}) and of cysteine on the uptake of NaI^{131} and of the major extraneous band solution by the thyroid gland of day-old chicks

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METHODS

Isolation of the extraneous band

NaI^{131} solutions (furnished by Abbott Radio-Pharmaceuticals, Oak Ridge, Tennessee or by E. R. Squibb and Sons, New Brunswick, New Jersey) were stored for two to three weeks at room temperature. Several microcuries of this solution were spotted on sheets of Whatman No. 1 filter paper (15 inches×15 inches) and developed one dimensionally in an ascending technique using n-Butanol saturated with 3N NH_4OH. After 20 hours, the chromatograms were air-dried and radioautographed. The location of the NaI^{131} band on the radioautograms was determined by running control strips spotted with a solution of 0.5 mg. NaI^{127} and developed under the same conditions. The NaI^{127} band was detected by dipping the strip in a solution of ammoniacal silver nitrate. The extraneous band, which moves slightly faster than the NaI^{131} band, was cut out in fifteen inch-long strips and the material was eluted off with 50% alcohol. The eluate was evaporated under nitrogen to a concentration of 5 microcuries/ml.

Uptake studies on chicks

Day-old chicks (New Hampshire Males×Single Comb White Leghorn Females) were obtained from fertile eggs received from the experimental poultry farms of Texas A and M College, College Station, Texas, or were purchased from Kazmeier-Sherrill Hatchery, Bryan, Texas. Chicks were given water, but no feed, and were injected with either 1 microcurie amounts of the extraneous band or with 1 microcurie of fresh solution of NaI^{131} (received from Abbott Radio-Pharmaceuticals, Oak Ridge, Tennessee, and found to contain over 90% of the radioactivity in the NaI^{131} band). At the end of 24 hours, the animals were drowned, their thyroids removed, and the collection of radioactivity was determined by the use of a well-counter.

RESULTS AND DISCUSSION

In the work reported previously (1) NaI^{131} was isolated by eluting the NaI^{131} band obtained from the paper chromatograms of stored NaI^{131} solutions. The eluate was then concentrated under nitrogen. Sodium iodide-I^{131} obtained by this procedure was rechromatographed and radioautographed. The results showed that 10 to 15% of the total radioactivity was present in the extraneous band probably due to the conversion of NaI^{131} into the extraneous band during elution, concentration and chromatography. However, several batches of fresh solutions of NaI^{131} (received from Abbott Radio-Pharmaceuticals, Oak Ridge, Tennessee) upon chromatography and radioautography showed over 90% of the radioactivity in the NaI^{131} band. Thus, studies were conducted to determine the uptake of fresh solutions of Abbott NaI^{131}, the NaI^{131} isolated from paper chromatograms of stored NaI^{131} solutions and the extraneous band solutions by the thyroid glands of day-old chicks obtained from Texas A and M eggs. It is apparent from the results of these studies presented in Table 1 that fresh solutions of Abbott NaI^{131} give about the same uptake as the NaI^{131} solutions isolated from paper chromatograms of stored NaI^{131}. Further, almost three times more radioactivity of the NaI^{131} solutions is collected in the thyroid gland as compared to the extraneous band solutions.

The results on the effect of iodine supplement in the diet of the pullets on

TABLE 1. EFFECT OF INJECTING FRESH SOLUTIONS OF ABBOTT NaI^{131} OR THE ISOLATED NaI^{131} SOLUTIONS OR THE EXTRANEOUS BAND SOLUTIONS UPON THE THYROID UPTAKE BY DAY-OLD CHICKS OBTAINED FROM TEXAS A AND M EGGS

Nature of I^{131} injected[1]	No. of chicks	% thyroid uptake of I^{131}
NaI^{131} fresh, Abbott	9	55 ± 5[2]
NaI^{131} isolated	5	56 ± 9
Extraneous band	9	22 ± 5

[1] Chicks were given water, but no feed and were injected intraperitoneally one microcurie of I^{131} (5 microcurie/ml.), and the thyroids were removed after 24 hours.
[2] Mean ± standard deviation of the mean.

the thyroid uptake of NaI^{131} by chicks obtained from these pullets are shown in Table 2. It is apparent from the results that the chicks obtained from pullets receiving 0.4 p.p.m. of iodine in the diet showed an uptake of 57%, while the chicks obtained from pullets receiving 4 p.p.m. iodine in the diet had an uptake of 9%. Rogler *et al.* (2) have reported an increase also in the NaI^{131} uptake by the embryonic thyroid in eggs from iodine-deficient hens. Since the chicks obtained from eggs furnished by A and M College of Texas showed a higher uptake of NaI^{131}, these were used throughout the present study.

The effect that different levels of iodine in the chicks' diets had on the thyroid uptake of NaI^{131} by 3-day old and 7-day old chicks was studied (Table 3). The results indicate no significant lowering of uptake of NaI^{131} by chicks maintained on water without food. There was a gradual decrease in the NaI^{131} uptake by chicks receiving an iodine-deficient diet whereas the chicks receiving Purina Growena, or the iodine-supplemented deficient diet, showed a rapid decrease in the NaI^{131} uptake by the thyroid gland.

The results on the effect of dosage of NaI^{131}, and of the extraneous band solution, upon the uptake by the thyroid gland of day-old chicks are presented in Table 4. From these results, it is apparent that the uptake of the extraneous band solution is lower than the NaI^{131} for all the three dosage levels tested.

The results on the effect of injecting NaI^{127} upon the thyroid uptake of NaI^{131} or of the extraneous band solution by day-old chicks are plotted in

TABLE 2. EFFECT OF IODIDE SUPPLEMENT IN PULLETS' DIETS ON THE THYROID UPTAKE OF NaI^{131} BY DAY-OLD CHICKS

Source of chicks	% iodine[1] in the diet of pullets	No. of chicks	% thyroid[2] uptake of NaI^{131}
Texas A and M College	0.00004	9	57 ± 6.1[3]
Kazmeier-Sherrill Hatchery	0.0004	9	9 ± 2.2

[1] All the iodine analyses throughout the present report were conducted by Dr. Carl Tiedcke, Laboratory of Microchemistry, 705 George Street, Teaneck, New Jersey.
[2] Chicks were injected intraperitoneally 1 microcurie of NaI^{131} (5 microcuries/ml.) and the thyroids were removed after 24 hours.
[3] Mean ± standard deviation of the mean.

TABLE 3. EFFECT OF IODIDE IN THE CHICKS' DIETS ON THE THYROID UPTAKE OF NaI^{131} BY CHICKS OBTAINED FROM TEXAS A AND M EGGS

Diet	% iodine in the diet	No. of chicks	% Thyroid[2] uptake of NaI^{131}		
			Initial	After 3 days	After 7 days
Starved	None	18	56 ± 2.7 (6)[3]	51 ± 8.8 (6)	DEAD
Purina Growena	0.00042	12		17 ± 6.1 (6)	8 ± 1.2 (6)
Iodine Deficient[1]	0.00006	12		46 ± 6.0 (6)	28 ± 1.2 (6)
Iodine Deficient[1] + 0.0005% iodine	0.00056	12		14 ± 7.0 (6)	7 ± 2.2 (6)

[1] Iodine deficient diet was obtained from Nutritional Biochemical Company, Cleveland, Ohio.

[2] Chicks were injected intraperitoneally 1 microcurie of NaI^{131} (5 microcuries/ml.) and the thyroids were removed after 24 hours.

[3] Mean ± standard deviation of the mean. Numbers in parentheses indicate the number of chicks.

Figure 1. One microcurie of NaI^{131} or of the extraneous band solution was injected intraperitoneally. Different levels of NaI^{127} were injected concomitantly but into different injection sites. The administration of in-

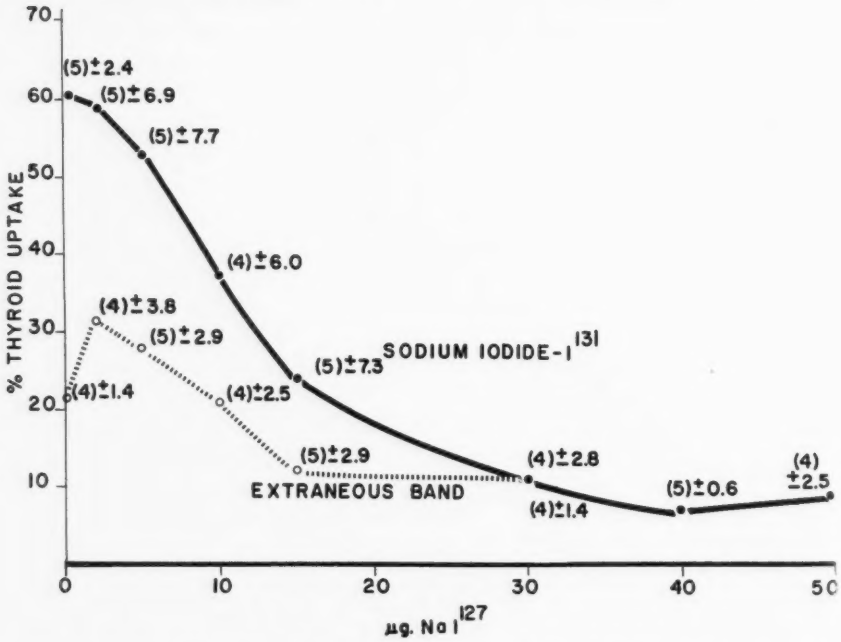

FIG. 1. Effect of injecting NaI^{127} on the thyroid uptake of NaI^{131} or the extraneous band by day-old chicks obtained from Texas A and M eggs. One microcurie of NaI^{131} or the extraneous band (5 microcurie/ml.) was injected intraperitoneally. NaI^{127} or cysteine HCl (Fig. 2) was injected intraperitoneally at the same time, but at different sites. The number in the parenthesis indicate the no. of chicks and ± the standard deviation of the mean.

TABLE 4. EFFECT OF DOSAGE OF NaI^{131} OR THE EXTRANEOUS BAND ON THE THYROID UPTAKE BY CHICKS OBTAINED FROM TEXAS A AND M EGGS

Amount of NaI^{131} injected	% thyroid uptake	Amount of extraneous band injected	% thyroid uptake
1 microcurie	56 ± 10.4 (3)[1]	1 microcurie	24 ± 1.0 (5)[2]
0.5 microcurie	53 ± 7.9 (4)	0.5 microcurie	23 ± 2.1 (5)
0.1 microcurie	55 ± 6.8 (4)	0.1 microcurie	23 ± 4.4 (5)

[1] Numbers in parentheses indicate the no. of day-old chicks.
[2] Mean ± standard deviation of the mean.

creasing levels of NaI^{127} decreases the uptake of NaI^{131}, while there is an initial rise in the uptake of the extraneous band solution when the chicks are injected with 2 to 5 μg. of NaI^{127}. The increase in uptake of the extraneous band solution obtained by the simultaneous injection of NaI^{127} may be due to a partial conversion of the extraneous band solution into NaI^{131} (1). Experiments were also conducted using 2 μg. of NaI^{127} injected 2 hours before the extraneous band solution. In this instance the uptake was increased to 29%.

The thyroid uptake of NaI^{131} or the extraneous band solution appears to be enhanced by the administration of 1 to 10 mg. of cysteine hydrochloride (Fig. 2). One microcurie of NaI^{131} or of the extraneous band solution was injected intraperitoneally and supplements of cysteine hydro-

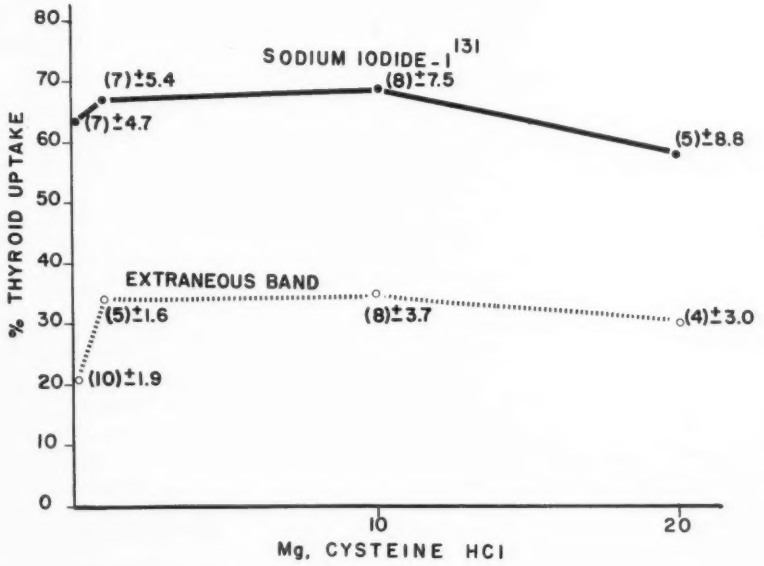

FIG. 2. Effect of injecting cysteine HCl on the thyroid uptake of NaI^{131} or the extraneous band by day-old chicks obtained from Texas A and M eggs. The numbers in parentheses indicate the no. of chicks and ± the standard deviation of the mean.

chloride were injected concomitantly but into different injection sites. Studies were conducted on the effect that injecting 20 mg. cysteine hydrochloride 2 hours before the administration of either NaI^{131} or of the extraneous band solution has upon the thyroid uptake by day-old chicks. The results showed the same uptake as obtained when both were administered simultaneously. The increase in uptake of NaI^{131} by the chicks receiving cysteine hydrochloride may be due to the sulfhydryl moiety of cysteine protecting the NaI^{131} from conversion to the extraneous band; however, the increase in the uptake of the extraneous band after administration of cysteine may be due to the partial conversion of the extraneous band into the NaI^{131} (1).

REFERENCES

1. Doctor, V. M. and J. B. Trunnell: *Endocrinology* **64:** 455. 1959.
2. Rogler, J. C., H. E. Parker, F. N. Andrews and C. W. Carrick: *Poultry Science* **38:** 405. 1959.

THE EFFECT OF BILATERAL ABLATION OF THE AMYGDALA ON ENDOCRINE FUNCTION IN THE RAT[1]

TAKASHI YAMADA AND MONTE A. GREER

Division of Endocrinology, Department of Medicine, University of Oregon Medical School, Portland, Oregon

ABSTRACT

Bilateral electrolytic destruction of the amygdala in male rats does not significantly interfere with thyrotropin or corticotropin secretion by the pituitary, but gonadotropin production appears definitely reduced. The amygdalectomized rats were adipsic and aphagic.

THERE is ample evidence at present to indicate that pituitary secretion is modulated by the central nervous system. Although most studies have concentrated on the hypothalamus, and there is convincing evidence that this area is of first rank importance in controlling hypophysial function, other areas of the brain have also been implicated. In particular, recent studies from various laboratories (1–7) have indicated that that part of the rhinencephalic-limbic system known as the amygdala is of importance to gonadotropic and corticotropic function.

Because of these reports and our continuing interest in the neural control of pituitary function, it was decided to investigate the effect of bilateral destruction of the amygdala on endocrine function in the rat, concentrating particularly on the secretion of thyrotropin.

MATERIALS AND METHODS

Adult male Holtzman rats were used. The amygdala was destroyed bilaterally by electrolytic lesions made with a stereotaxic instrument (8). Thirty separate electrode insertions were performed in each animal and a direct current of 6 ma. for 15 seconds was utilized with each insertion. Five days postoperatively 30 mg. of propylthiouracil (PTU) in 10% gum acacia were injected subcutaneously daily at rotated sites. A second group of animals received PTU but had no lesions, while a third group served as untreated controls.

All force-fed animals were given a fluid mixture containing 200 parts canned milk, 100 parts Karo syrup and 12 parts essential amino acid mixture (Essenamine®, Winthrop-Stearns) twice daily by stomach tube. A control-fed group of rats had its diet restricted so that the weight curve approximated that of the amygdalectomized group.

At autopsy, thyroid, adrenals, thymus, and testes were dissected and weighed on a torsion balance, then fixed in 10% formalin. The brains and pituitaries of the amygdalectomized rats were fixed in situ in the unroofed skull. Serial sections of the brain were

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made at 10 μ and every 10th section was mounted and stained with toluidine blue. Testes were sectioned at 10 μ and stained with H & E.

RESULTS

Following destruction of the amygdala the animals stopped eating and drinking completely, possibly because of some extension of the lesions to the lateral hypothalamus wherein are located the "feeding" and "drinking" centers. Because of this it was necessary to force-feed the animals twice daily and to inject them with 10–15 ml. of physiologic saline daily intraperitoneally. On days 6–8, force-feeding and saline injections were discontinued to see if the animals would resume normal eating and drinking behavior. The rapid weight loss associated with their failure to do so (Fig. 1) necessitated resumption of gavage and saline.

Not only did the animals stop eating and drinking but they also lost all interest in esthetic values. They stopped cleaning themselves and their coats and tails became matted with thick brownish material and remnants of feces. They were quite docile throughout the experiment but not notice-

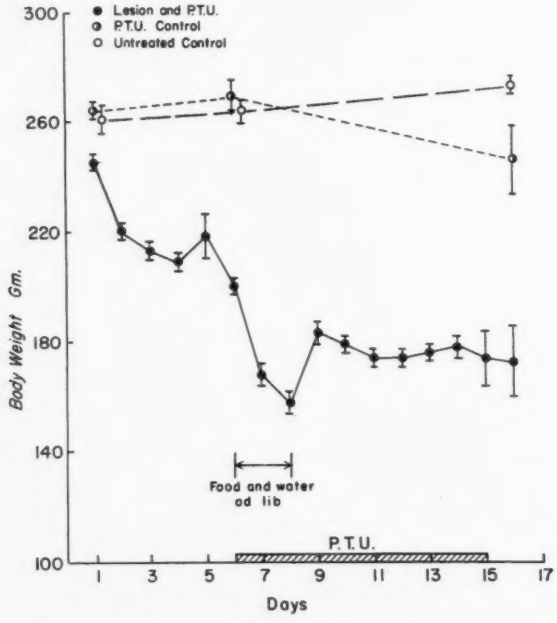


FIG. 1. Weight curves of experimental animals, expressed as mean and standard error. PTU = propylthiouracil, 30 mg. daily subcutaneously. Lesion = bilateral electrolytic destruction of the amygdala. The amygdalectomized group was force-fed a synthetic diet; the other two groups were fed Purina laboratory chow *ad lib.* The "Lesion and PTU" group contained 11 animals, the "PTU Control" Group 5 animals, and the "Untreated Control" Group 7 animals. Amygdalectomy was on day 1.

ably more so than the control animals. However, they did appear to exhibit less spontaneous movement than the control rats. There was no evidence of "sham rage" or hypersexuality.

Because the lesioned animals lost some weight in spite of vigorous exogenous dietary care (Fig. 1), organ/body weight is expressed in reference to the weight of the animals at the start of the experiment rather than at autopsy. Some of the experimental animals failed to survive until the originally scheduled day of autopsy.

Thyroid weight averaged about 5 mg./100 gm. in the untreated control group but increased to 10–14 mg. after 9–10 days administration of PTU

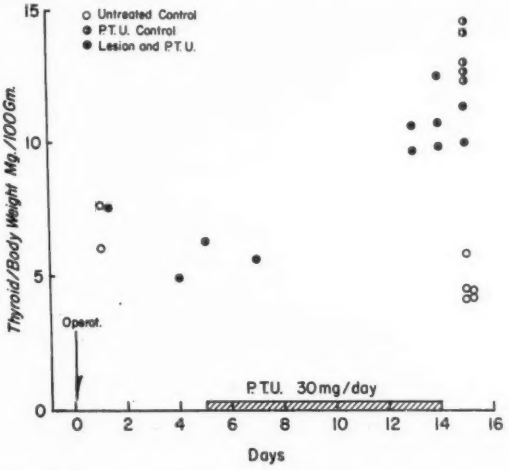


FIG. 2. Thyroid weights at autopsy of the 3 experimental groups in Figure 1. Thyroid /body weight is expressed in reference to the weight of the animals at the start of the experiment.

in both the lesioned and control animals (Fig. 2). The thyroids were slightly smaller in the lesioned animals, but when calculated on the basis of final body weight there was no difference between the thyroid weights of the lesioned and control animals.

The adrenal glands in the lesioned rats gradually increased in size after surgery and reached a maximum value about 13 days postoperatively, then declined slightly (Fig. 3). Some reservation must be attached to the interpretation of the apparent curve of change in adrenal size since the number of animals involved is rather small. Thymic involution was noted in all lesioned animals.

An interesting finding, particularly when related to earlier studies on reproductive changes caused by stimulation or destruction of the amygdala was the gradual decrease of the weight of the testes in our experimental animals when compared with both control groups (Fig. 4, 5). Microscopi-

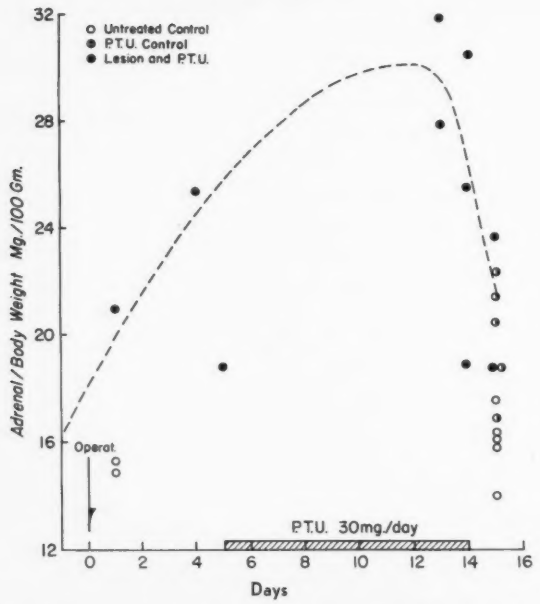


FIG. 3. Adrenal weights at autopsy of the 3 experimental groups in Figure 1. Adrenal /body weight is expressed in reference to the weight of the animals at the start of the experiment.

cally they showed considerable tubular atrophy with greatly reduced formation of spermatozoa and a slight reduction in the number of Leydig cells (Fig. 6, 7). These changes were not as marked as those found in rats hypophysectomized for a comparable length of time (Fig. 8). The diet-restricted control rats did not exhibit this rather pronounced testicular atrophy (Fig. 9).

Brain sections showed complete or almost complete destruction of the entire amygdaloid complex bilaterally, with only slight encroachment of the lesions in some animals into the lateral hypothalamus (Fig. 10, A–D). The pituitaries were not damaged, although no specific cytologic stains were made.

DISCUSSION

Previous studies involving destruction or electrical stimulation of the amygdala have indicated that this complex is of some importance in the control of gonadotropic activity of the pituitary gland (1–5). In the present investigation, it was found that the testes were much smaller in the lesioned animals than in either the PTU or untreated control groups. It is well known that malnutrition will cause a decrease in gonadotropic activity of the pituitary (9, 10) and thus the decreased testis size might be related

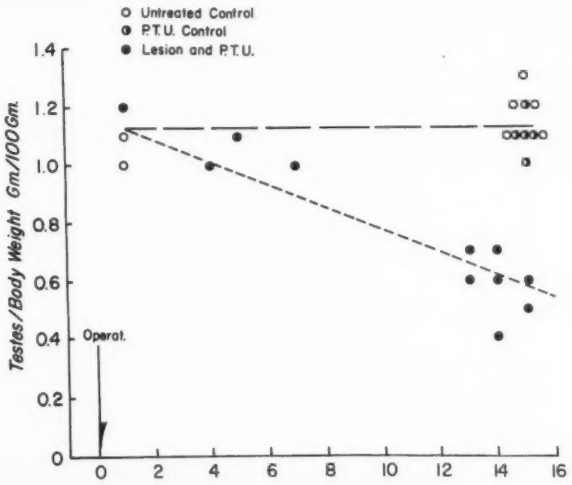


FIG. 4. Weight of testes at autopsy of the 3 experimental groups in Figure 1. Testes /body weight is expressed in reference to the weight of the animals at the start of the experiment.

FIG. 5. Testes and seminal vesicles of 16-day postoperative amygdalectomized rat on the left compared with those of a normal control on the right.

FIG. 6. Testis of normal control rat (×105).

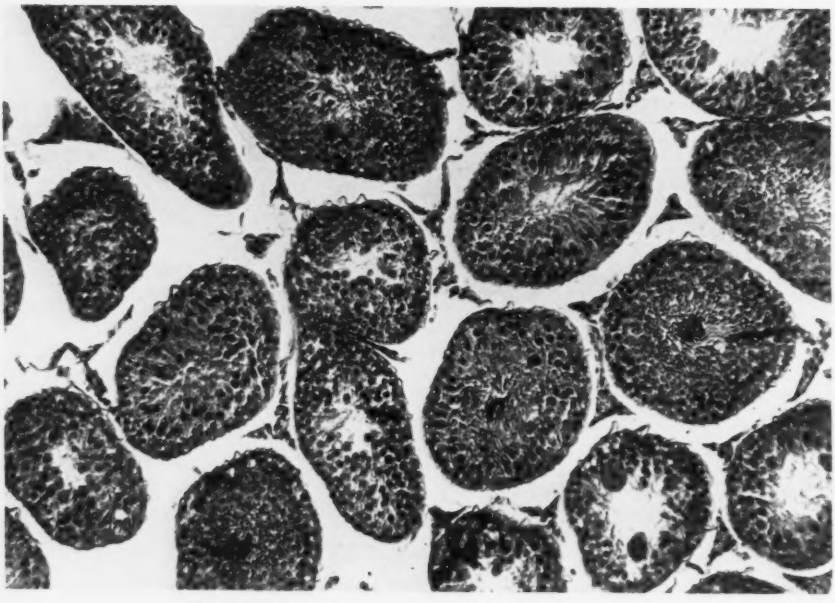


FIG. 7. Testis of amygdalectomized rat (×105).

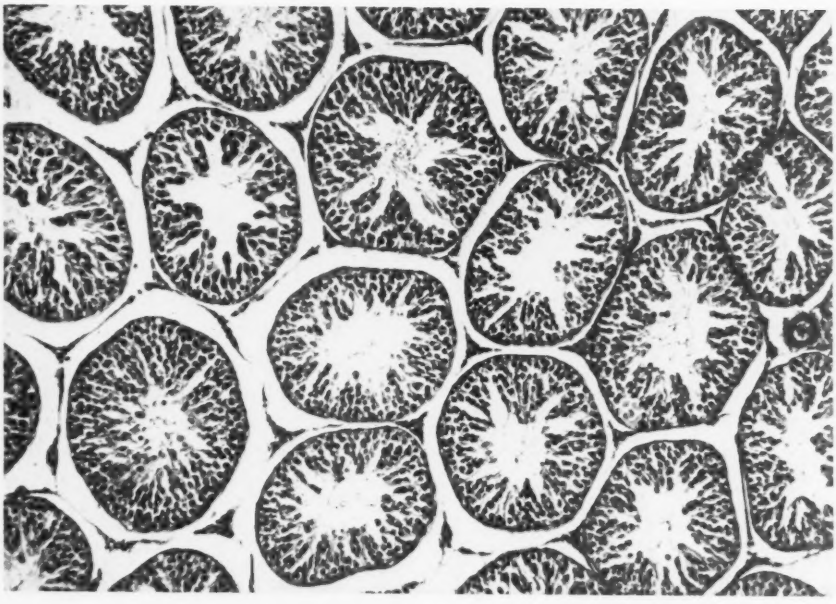


FIG. 8. Testis of rat hypophysectomized for 14 days (×105).

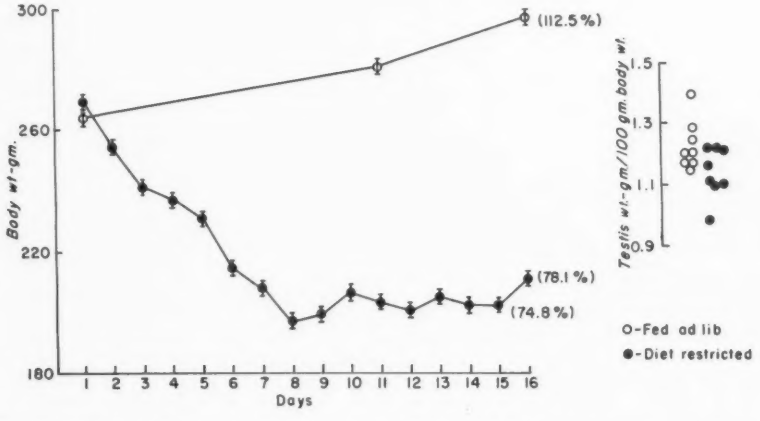
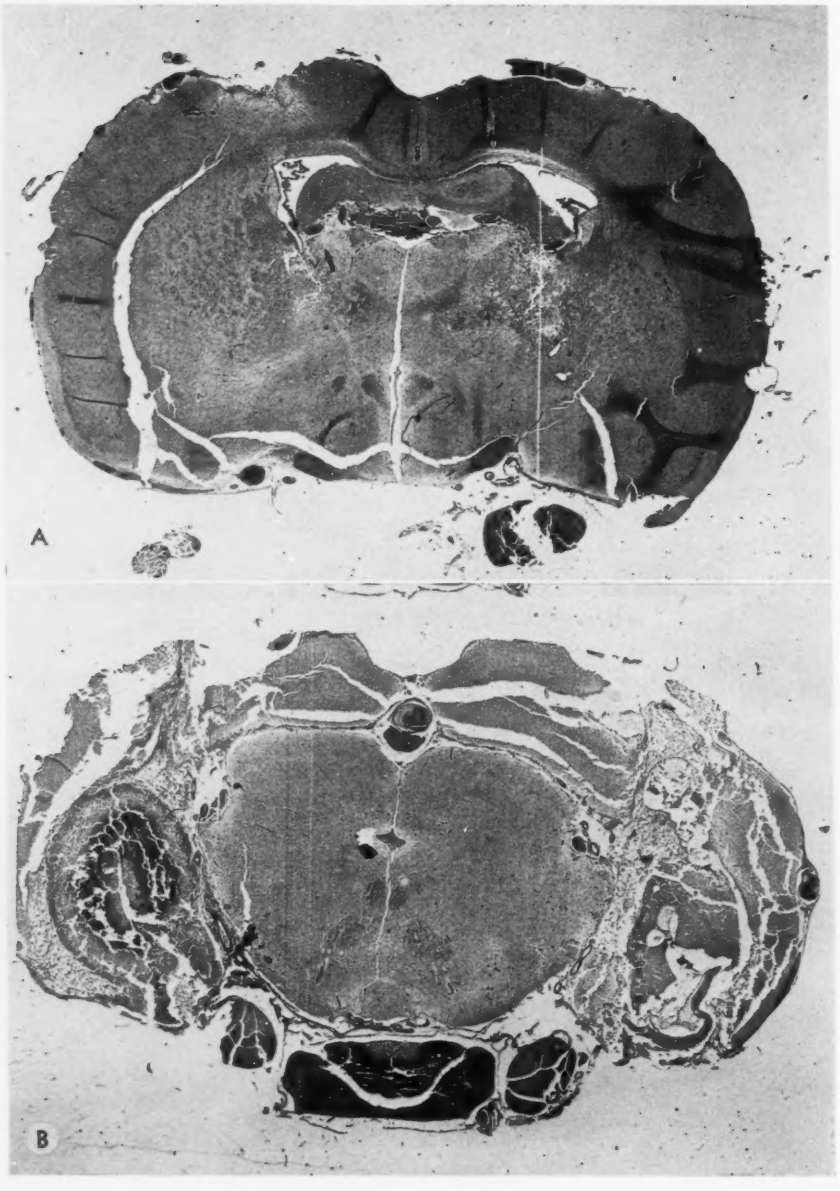


FIG. 9. Body and testis weight of rats on a diet restricted so as to make their weight curve similar to that of the amygdalectomized animals compared with their normal *ad lib.*-fed controls. Figures in parentheses compare body weight with that at the beginning of the experiment.


A
B

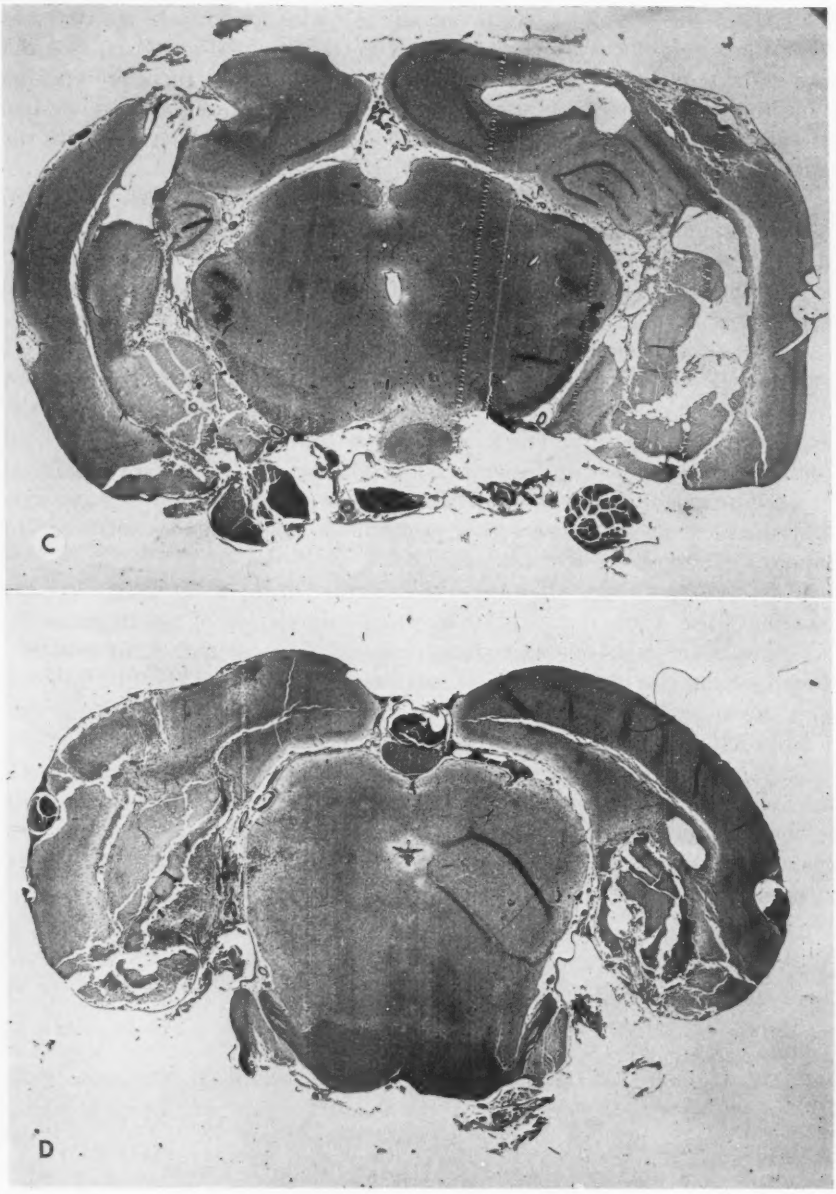


FIG. 10. Frontal brain sections of a rat with the amygdala destroyed by bilateral electrolytic lesions. A. Level of the paraventricular nucleus of the hypothalamus; B. Level of the mid-pituitary; C. Level of the mammillary bodies; D. Level of the pons. All sections magnified 10×.

to this non-specific effect. However, animals with food intake restricted so that their weight curves corresponded to the amygdalectomized rats did not show any significant reduction in testicular size. It therefore appears plausible that the testicular atrophy reported herein might be related to the absence of a functioning amygdaloid complex which is necessary for the adequate secretion of gonadotropins.

Two possible alternatives might be suggested as a cause of the increased adrenal weight in the lesioned animals: (1) The hypertrophy was merely due to operative stress and treatment with PTU and had no specific relation to the lesions in the amygdala. (2) Certain neural centers destroyed by the lesions normally inhibit ACTH production and their destruction resulted in an increased secretion of ACTH by the pituitary.

Since the adrenals became smaller after reaching a maximal value, we feel that the increased adrenal size was probably a non-specific result of the experimental procedure. The adrenal hypertrophy was apparently related to increased adrenocortical secretion since thymic involution was observed in all lesioned animals. It is of interest that Martin *et al.* (6) have also described increased adrenocortical secretion in dogs and cats subjected to bilateral removal of the amygdala.

Mason (7) has found that a marked increase in the 17-hydroxycorticoid content of the serum occurs after electrical stimulation of the amygdala in primates. Stimulation of contiguous areas in his experiments did not produce a similar rise of blood corticoids. However, the present results indicate that the amygdala is not essential for increased ACTH release in the rat.

Thyrotropin secretion appeared to be little affected by destruction of the amygdala as the goitrogenic response was approximately the same in both the lesioned and control animals administered PTU. This observation adds to the accumulating body of evidence that destruction of central nervous system centers other than the anterior hypothalamus does not appreciably interfere with thyrotropin secretion.

REFERENCES

1. Alonso-Deflorida, F. and J. M. Delgado: *Am. J. Physiol.* **193:** 223. 1958.
2. Faure, J., R. Quilichini and J. Gueyne: *J. Physiol., Par.* **48:** 531. 1956.
3. Koikegami, H., T. Yamada and K. Usui: *Folia psychiat. et neurol. japonica* **8:** 7. 1954.
4. Koikegami, H., S. Fuse, T. Yokoyama, T. Watanabe and H. Watanabe: *Folia psychiat. et neurol. japonica* **8:** 336. 1954.
5. Shealy, C. N. and T. L. Peele: *J. Neurophysiol.* **20:** 125. 1957.
6. Martin, J., E. Endroczi and G. Bata: *Acta physiol. hung.* **14:** 131. 1958.
7. Mason, J. W.: *Am. J. Physiol.* **196:** 44. 1959.
8. Greer, M. A., C. Mencken and K. T. Bolen: *Proc. Soc. Exper. Biol. & Med.* **89:** 480. 1955.
9. Mulinos, M. G. and L. Pomerantz: *J. Nutrit.* **19:** 493. 1940.
10. Stephens, D. J. and W. M. Allen: *Endocrinology* **28:** 580. 1941.

PURIFICATION OF SHEEP PITUITARY FOLLICLE-STIMULATING HORMONE (FSH) BY ION EXCHANGE CHROMATOGRAPHY ON DIETHYLAMINOETHYL (DEAE)-CELLULOSE[1]

M. C. WOODS AND M. E. SIMPSON

Institute of Experimental Biology and The Department of Anatomy, University of California School of Medicine, San Francisco, California

ABSTRACT

Preparations of sheep FSH have been made by extraction of ground whole pituitaries with 40% ethanol, fractionation with $(NH_4)_2SO_4$, and anion exchange column chromatography on DEAE-cellulose at pH 7 in phosphate buffer. The most advantageous removal from the column of biologically active contaminants and the bulk of the protein was with 0.030M phosphate buffer. Thereafter the FSH was removed optimally with 0.060M phosphate. Subsequent refractionation with $(NH_4)_2SO_4$ further increased the potency of FSH to a minimal effective dose (MED) of 1.7 μg. (65 times the potency of Armour Standard FSH 264–151X). This final FSH preparation showed contamination with ICSH (LH) judged by repair of interstitial tissue in the hypophysectomized female rat at 70 times this MED, and at 85 times the MED judged by response of the ventral prostate (100% increase in weight) in immature hypophysectomized male rats. The follicle-stimulating activity as well as this ratio between FSH and ICSH was improved above any previously reported for preparations from sheep pituitary. Evidence of estrogen secretion was seen at much lower levels than interstitial repair; estrous distension occurred at 15 times the MED for FSH; luteinization of follicles occurred at a dosage 20 times the MED for FSH.

ALTHOUGH considerable progress has been reported during the past 20 years in the purification of sheep pituitary FSH, there is a continuing need to improve both the chemical and biological purity. Preparations have been described varying in minimal effective doses in hypophysectomized rats from 50–100 μg. (1, 2) to 2.5–3.0 μg. (3–5). The preparations with greatest potency were physico-chemically heterogeneous, while less potent ones were reported to be homogeneous (1, 6). Attention has been called by Raacke *et al.* (7) to the unreliability of physico-chemical criteria in judgement of ultimate purity of the latter preparations; on further purification the hormonal activity was not found associated with the main protein component.

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The principal biologically active contaminant of FSH has been interstitial cell stimulating hormone (ICSH or LH). The presence of ICSH was shown when preparations from sheep pituitary were administered in dosages 10 to 40 times the MED for FSH (3, 8, 9).

The earlier efforts to purify sheep FSH utilized techniques of isoelectric precipitation and differential solubility in salt solutions and organic solvents (1, 3, 8–11). Recent attempts have applied ion exchange adsorbents Amberlite IRC-50 (12, 13), diethylaminoethyl (DEAE)-cellulose (4, 5, 14, 15), and hydroxylapatite (5). The present communication deals with the use of DEAE-cellulose in the purification of sheep FSH previously concentrated and fractionated by ammonium sulfate precipitation (15).

METHODS

Bioassay procedures

Both in the biological standardization for FSH potency and for other pituitary hormones as contaminants use was made of immature hypophysectomized rats of the Long-Evans strain. FSH was determined by the method of Evans *et al.* (16, 17).[2] Comparisons of FSH potency were also made with Armour Standard FSH 264-151X and NIH-FSH-S1.[3] The ICSH contamination of the purified FSH preparations was determined by assay in female rats by the method of Evans *et al.* (16), and also in male rats by the ventral prostate method of Greep *et al.* (19, 20).[4] Comparisons of ICSH potency were

[2] The MED for FSH was the minimal total dose among a series of graded dosages administered subcutaneously to hypophysectomized immature female rats which during 72 hours caused microscopically measurable follicular development with beginning antrum formation (an increase from a follicular diameter of 375 μ to 450–500 μ) in two-thirds of the rats tested. The data obtained from the dose range which initiated follicular development in 0 to 100% of the experimental group (average 9 rats per dose level) were analyzed statistically as a quantal response (18). Dosage was regarded as the independent variable and the per cent of individuals at each dose which had follicular development as the dependent variable. In this situation, a normal distribution of log-threshold dose was found to apply. The effective dose for follicular development in 67% of the test animals (ED67), as determined graphically from the plot of the probit of per cent response to the log-dose, was found to correspond to the MED commonly determined by empirical observation. The potencies (MED) for FSH reported in this paper were checked graphically as the ED67, and the confidence limits were determined at a lower limit of 5% response (ED5) and an upper limit of 95% response (ED95). The index of precision (the reciprocal of the slope of probit plotted against log-dose) for eleven purified FSH preparations averaged lambda = 0.061 (range 0.038 to 0.082).

[3] Armour Standard FSH 264-151X was provided through the courtesy of the Biochemistry Research Department, Armour Pharmaceutical Company. NIH-FSH-S1 was a gift of the Endocrinology Study Section, National Institutes of Health, Bethesda, Md.

[4] Contamination with ICSH was considered to be present at the minimal dose among a series of graded dosages administered intraperitoneally to hypophysectomized female rats which during 72 hours caused repair of ovarian interstitial tissue (nuclear enlargement with loss of pyknosis, and expansion of cytoplasm with increased eosinophilia) in two-thirds of the rats tested. As in the determination of the MED for FSH, the assay data for ICSH were submitted to probit analysis. The ED67 was determined to coincide with the empirical MED. In this case the probit of the per cent of individuals responding with interstitial cell repair was plotted against the log-dose. Confidence limits were

made with Armour LH 227-80.[5] Growth hormone was determined by the tibia test (22, 23). Thyrotrophic (TSH) and adrenocorticotrophic (ACTH) hormonal activities were determined histologically (24, 25). Lactogenic hormone was determined by the local crop sac method in pigeons (26). Antagonism to gonadotrophins was tested by the methods previously reported (16, 27).

Chemical procedures

The extraction of acetone-dried sheep pituitaries with 40% ethanol was carried out at 25 to 27° C. All other fractionation procedures were conducted in a refrigerated bath or cold room at 3 to 5° C. Fractions from solvent or salt precipitation or from chromatographic separation were recovered by lyophilization following dialysis. Yields were calculated on a dry-weight, salt-free basis. Protein was determined by ultraviolet spectrophotometry relative to the starting protein fraction so that recovery of fractions from chromatography was estimated in terms of the per cent of total ultraviolet (276 mμ.)-absorbing material applied to the column. Fractionation with $(NH_4)_2SO_4$ was made at pH 5.0 to 5.3. Saturated $(NH_4)_2SO_4$ solution was added dropwise to aqueous protein solutions to produce a concentration of 2.0M $(NH_4)_2SO_4$; solid $(NH_4)_2SO_4$ was added above 2.0M. The concentrations of $(NH_4)_2SO_4$ were corrected for volume expansion. Diethylaminoethyl (DEAE)-cellulose which contained 0.45 m. eq. basic groups per gm. was prepared according to Ellis and Simpson (28). Columns of DEAE-cellulose were prepared for chromatography by washing at room temperature successively with 1N NaOH, distilled water, and 0.005M sodium phosphate buffer (pH. 7) to an effluent pH of 7.0; subsequently the columns were thoroughly equilibrated with the same buffer at 3 to 5° C. Proteinase I activity was measured by the method of Adams and Smith (29) with activation of enzyme as reported by Ellis (14).

RESULTS

The FSH potency of whole, frozen sheep pituitaries was first concentrated 150 fold by the use of $(NH_4)_2SO_4$ by methods essentially those reported by Jensen *et al.* (8) and Fraenkel-Conrat *et al.* (30). The tissue was ground, dried with acetone and extracted with 40% ethanol. The 40% ethanol extract[6] was fractionated to recover successively the material which was soluble in water (pH 4.0 to 4.8), insoluble in 60% acetone (pH 5.2), soluble in 0.2M NaCl (pH 6.5 to 7.0), and insoluble in $(NH_4)_2SO_4$ between the limits of 2.0 and 3.0M. The latter fraction was subjected three additional times to fractionation between 2.0 and 3.0M $(NH_4)_2SO_4$, keeping the protein concentration constant at 0.75% protein when in 2.0M

established between ED5 and ED95. The average index of precision for sixteen preparations was lambda = 0.084 (range 0.040 to 0.150).

In immature hypophysectomized male rats, an increase of 100% in the weight of the ventral prostate was considered as significant evidence for the presence of ICSH (20).

[5] Armour LH 227-80 was provided through the courtesy of the Biochemistry Research Department, Armour Pharmaceutical Company.

[6] The average yield from 40% ethanol extraction was 35 gm. per kg. of acetone-dried tissue (8 gm. per kg. of original wet tissue). The MED for FSH and ICSH was the same: 125 to 250 μg. Two mg. contained 0.15 IU lactogenic hormone; contamination with growth and thyrotrophic hormones was approximately 0.1%, with ACTH less than 0.1%.

$(NH_4)_2SO_4$ solution. During the procedure the fractions precipitating in 2.0M $(NH_4)_2SO_4$ and remaining insoluble during dialysis were discarded. The yield of the final fraction was 1.4 to 1.8 gm. per kg. of acetone-dried glands. The MED for FSH was 14 μg.; the MED for ICSH was 75 μg. This preparation contained 20 to 25% of the FSH activity of original tissue. Contamination with each of the other anterior pituitary hormones (growth, lactogenic, thyrotrophic and adrenocorticotrophic) was less than 0.1%.

Anion exchange column chromatography on DEAE-cellulose achieved a further 3 to 6 fold increase in FSH potency. Such fractions contained 8 to 11% of the dry-weight or of the ultraviolet-absorbing material applied to the column. As shown in the chromatograms in Figure 1, the FSH preparation was applied to the column in 0.005M phosphate buffer (pH 7.0) at a protein load of 15 mg. per ml. of adsorbent. The unadsorbed material was collected and pooled as fraction A. The second fraction (B) was removed from the column by elution with phosphate buffer at either 0.020M, 0.025M or 0.030M phosphate buffer. Fractions C and D were eluted with 0.060M and 0.080M phosphate buffer respectively. Table 1 shows that the column retained all of the FSH activity at 0.005M phosphate and that 1% or less of the FSH applied could still be recovered in fraction D. The B-fractions contained an additional 5 to 10% of the FSH activity applied to the column. The C-fractions contained the major portion, 50 to 65%, of the FSH activity and 10 to 12% of the dry weight applied. The FSH potency of the C-fraction and its contamination with ICSH were dependent on the concentration of phosphate with which the B-fraction was removed. The most favorable condition for elution of fraction B was found to be use of 0.030M phosphate, which permitted the recovery of FSH with MED of 2.9 μg. (III-72C). The MED for ICSH in this fraction was approximately 35 times the minimal dose for FSH. After elution with either 0.025 or 0.030M phos-

TABLE 1. YIELD AND GONADOTROPHIC POTENCY OF FRACTIONS OBTAINED FROM DEAE-CELLULOSE CHROMATOGRAPHY STARTING WITH FSH PURIFIED BY $(NH_4)_2SO_4$ FRACTIONATION

(I–111, MED* FSH 13.7 (10.0–16.1) μg., MED* ICSH 75 (65–85) μg.)

Fraction No.	Concentration of eluting buffer M	Yield		MED*		
				FSH		
		mg./kg. of acetone-dried tissue	% of FSH in I-111	μg.	Potency compared to Armour 151X**	ICSH μg.
III-40A	0.005	600	< 1	> 500		260 (160–290)
III-40B	0.020	170	5	50 (30– 58)		450 (300–490)
III-40C	0.060	175	50	4.2 (3.1– 5.1)	25×	45 (28– 55)
III-40D	0.080	60	1	65 (52– 75)		>1000
III-25A	0.005	600	< 1	> 500		260 (160–290)
III-25B	0.025	150	10	14.3 (11.5–17.0)		50 (30– 60)
III-25C	0.060	150	60	4.3 (3.1– 5.1)	25×	95 (55–115)
III-25D	0.080	70	< 1	250		>250
III-72A	0.005	585	< 0.1	>6000		310 (270–375)
III-72B	0.030	170	10	17.0 (14.0–19.0)		165 (135–400)
III-72C	0.060	130	65	2.9 (2.2– 3.6)	40×	105 (55–145)

* MED corresponds to ED67 from probital analysis. Confidence interval (ED5–ED95) is included in parentheses.
** In hypophysectomized female rats, Armour Standard FSH 264-151X had an MED for FSH of 110 (95–125) μg. MED for ICSH contamination was 130 (90–150) μg. (According to Steelman and Pohley (31), this Armour FSH Standard contained 1% of the LH activity of Armour LH 227-80 judged by the ventral prostate test.)

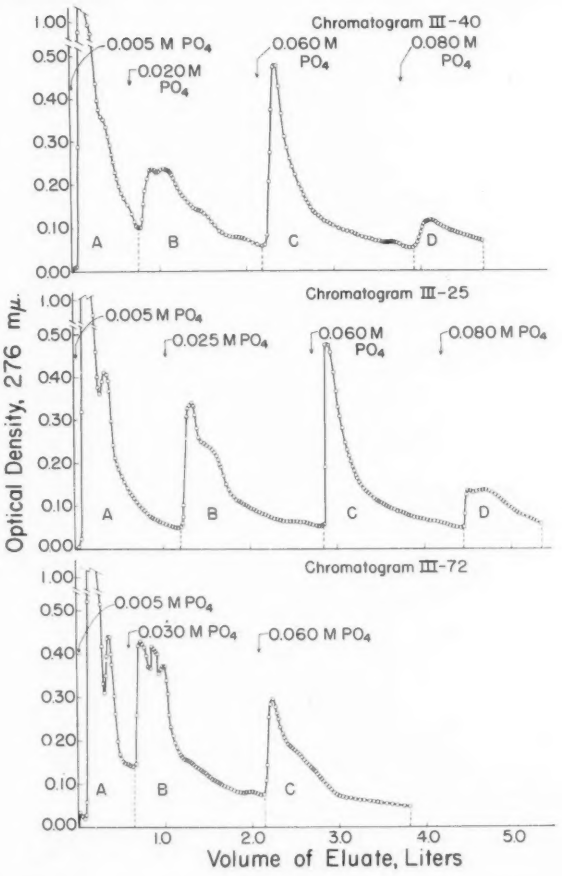

FIG. 1. Column chromatography on DEAE-cellulose of sheep FSH concentrated by $(NH_4)_2SO_4$ fractionation. 2.0 gm. quantities of fraction I–111 (MED FSH 14 μg., ICSH 75 μg.) in 20 ml. of 0.005M sodium phosphate buffer, pH 7.0, were applied in each case. Column dimensions were 2.0×40 cm. Elution of zones as indicated was accomplished at +3° C. with sodium phosphate buffers, pH 7.0, at a flow rate of 9.5 to 11 ml. $hr.^{-1}$ $cm.^{-2}$.

phate, contamination of the C-fractions was less than 0.1% with growth, lactogenic, thyrotrophic or adrenocorticotrophic hormones.

Rechromatography of C-fractions with MED for FSH of 5 μg. on DEAE-cellulose using gradient elution with 0.1M phosphate buffer (pH 7.0) showed that the product at this stage of purification was chromatographically heterogeneous. Considerable loss of FSH activity resulted from rechromatography and with no improvement in the specific activity of FSH; only 20% of the total applied activity was recovered.

An additional 2 fold concentration of FSH activity in C-fractions was accomplished by refractionation in dilute aqueous solutions (0.075%) by $(NH_4)_2SO_4$, using a procedure essentially the same as described by Fraenkel-Conrat *et al.* (3). The yields and gonadotrophic potencies recovered from C-fractions by precipitation at 2.4M and 3.0M $(NH_4)_2SO_4$ are shown in Table 2. The FSH potencies in the fractions soluble in 2.4M but insoluble in 3.0M $(NH_4)_2SO_4$ were approximately doubled and the contamination with ICSH was halved; 7 to 14% of the FSH potency of the original tissue was recovered in the 3.0M $(NH_4)_2SO_4$-insoluble fractions. A fraction was obtained (IV-19F) having a MED for FSH of 1.7 μg., which represented a 1600 fold concentration of FSH activity of the original tissue; the yield was 55 mg. per kg. of acetone-dried tissue (14 mg. per kg. wet tissue). The

TABLE 2. YIELDS AND GONADOTROPHIC POTENCIES DERIVED BY $(NH_4)_2SO_4$ FRACTIONATION OF THE C-FRACTIONS FROM DEAE-CELLULOSE CHROMATOGRAPHY OF SHEEP FSH

Derived from fraction		Insoluble in $(NH_4)_2SO_4$		Per cent yield of starting fraction		Gonadotrophic potency					
						FSH			ICSH (LH)		
							Potency compared with			Potency compared with Armour LH 227-80††	
No.	MED μg.	2.4M	3.0M	Weight	FSH potency	MED* μg.	Armour 151X	NIH-FSH-S1†	MED* (IP) μg.	Interstitial repair (IP)	100% Increase ventral prostate weight (subc.)
III-40C	4.2	IV-10E		25	5	5.0 (3.8–5.8)			> 50		
			IV-10F	45	95	2.3 (1.9–2.8)	50×	25×	35 (25– 40)	0.13×	0.20×
III-25C	4.3	IV- 7E		25	10	5.0 (3.8–5.8)			40 (25– 60)		
			IV- 7F	45**	90	2.6 (2.1–3.2)	42×	21×	28 (20– 35)	0.16×	0.12×
III-72C	2.9	IV-19E		15	10	3.6 (2.5–4.2)			160 (115–200)		
			IV-19F	40	65	1.7 (1.2–2.1)	65×***	32×***	115 (65–180)	0.04×	0.10×

* MED corresponds to ED67 from probital analysis. Confidence interval (ED5–ED67) is included in parentheses.

** A remaining 20% (by weight) which was soluble in 3.0M $(NH_4)_2SO_4$ was recovered by full saturation with $(NH_4)_2SO_4$ and was found to be devoid of FSH activity.

*** Augmentation bioassay in Long-Evans rats under the conditions of Steelman and Pohley (31) showed this fraction to have 70 times the potency of Armour Standard FSH 264-151X and 31 times the potency of NIH-FSH-S1 as determined simultaneously.

† In hypophysectomized female rats, NIH-FSH-S1 had a MED for FSH of 55 (35–65) μg. and the MED for ICSH contamination was 250 (140–290) μg.

†† Judged by assay in hypophysectomized female rats, the MED for ICSH of Armour LH 227-80 was 4.6 (3.6–5.5) μg. The ventral prostate of hypophysectomized male rats was enlarged 100% at 13 μg., confirming Steelman and Pohley (31).

MED for ICSH was 115 μg. This preparation was contaminated less than 0.1% with growth, lactogenic, thyrotrophic or adrenocorticotrophic hormones. At all stages of purification of the 40% ethanol extract only traces of Proteinase I activity were demonstrable, even after incubation for 24 hours.

Remarkable stability of FSH has been observed in follicle-stimulating preparations at all degrees of purity. A preparation with MED for FSH of 20 μg. made by the method of Jensen *et al.* (8) was stored at room temperature for 20 years with no detectable loss of FSH potency. Fraction III-25C (MED for FSH 4.3 μg.) when stored for 10 months either in the lyophilized form, or in 10% aqueous solution in 0.9% NaCl-2.0% n-butanol both at 5° C or when frozen with occasional thawing and refreezing, also lost no potency. Fractions IV-10F and IV-7F (MED for FSH of 2.3 and 2.6 μg.) were stored in the lyophilized form at room temperature for 8

months without loss of FSH potency. The potency of fraction IV-19F was determined repeatedly during a period of 7 months and the potency remained at 1.7 μg. Steelman and Segaloff (4) found 40% loss of potency of lyophilized sheep FSH after storage for 1 month, and of 75% after 3 months.

Only 60 to 75% of the total FSH activity applied to the DEAE-cellulose columns was recovered during the chromatographic separations reported in the three experiments presented in Table 1. When rechromatographed, only 20% of the initial activity was recovered. Since there was no indication of loss of FSH potency on dialysis, lyophilization, or storage, this destruction apparently occurred on the column. Loss of activity on rechromatography of pig (4) and human (31) FSH on DEAE-cellulose has been reported.

TABLE 3. RESPONSE IN IMMATURE HYPOPHYSECTOMIZED LONG-EVANS RATS TO MULTIPLES OF THE MED FOR SHEEP FSH

Purification procedure	Fraction	MED* for FSH μg.	Multiple of MED**				
			Repair of interstitial tissue (MED ICSH)	Uterus		Scattering and luteinization of granulosa	100% Increase ventral prostate weight
				Significant weight increase	Estrous distension		
$(NH_4)_2SO_4$ Fractionation	I-111	13.7	5	3	5	10–20	20
DEAE-Cellulose	III-40C	4.2	11	5	9	12	35
	III-25C	4.3	22	6	10	9	30
	III-72C	2.9	35	7	9	9	26
$(NH_4)_2SO_4$ Following DEAE-Cellulose	IV-10F	2.3	15	3	5	8	30
	IV- 7F	2.6	12	4	10	10	45
	IV-19F	1.7	70	5–8	15	20	85

* MED corresponds to ED67 from probital analysis.
** All injections were intraperitoneal except for the ventral prostate test which were subcutaneous.

The biological response of hypophysectomized rats to high doses of the main FSH-containing fractions during progressive purification is shown in Table 3. As the MED for FSH was reduced from 14 μg. to 1.7 μg. a significant increase occurred in the multiple of the MED which repaired interstitial tissue (an increase from 5 to 70 fold); the multiple of the MED which was needed to double the ventral prostate weight was increased from 20 fold to 85 fold. Less improvement occurred in the multiple of the MED which produced uterine enlargement, estrous distension, scattering of granulosa or beginning luteinization of follicles. The theca interna of growing follicles was not thickened (epithelioid). Compact corpora lutea or thecal luteinization were rarely encountered up to doses 100 to 200 times the MED for FSH.

Although it has been the practice to standardize FSH by subcutaneous injection, due to the greater potency by this route of administration, it was found that all preparations with MED between 14 and 1.7 μg. (Table 3), were equally potent when administered intraperitoneally. Ovarian weights of hypophysectomized rats were usually increased 100% at the

MED for FSH, but this increase was not consistent enough to serve as a basis for assays. FSH fractions dissolved in 0.9% NaCl containing 0.1% bovine serum albumin did not cause an ovarian response at a lower dose than when dissolved in 0.9% NaCl. Injection twice daily instead of once also did not increase the effectiveness. FSH fractions with MED 14, 4 or 1.7 μg. injected intraperitoneally exerted no antagonism to other primary gonadotrophic stimuli[7] injected simultaneously by subcutaneous route.

DISCUSSION

The preparation of sheep FSH with a MED of 1.7 μg., and a potency 65 times Armour Standard FSH 264-151X, represents a significant advance in the purification of this hormone. The sheep FSH prepared by Jensen *et al.* (8) had a MED of 25 μg. Fraction I-111 (Table 3) of the present procedures was prepared similarly and had approximately equivalent potency. The FSH of Li *et al.* (1, 2) had a MED of 50 to 100 μg.; this fraction was reported by Steelman and Pohley (32) to be equipotent by augmentation bioassay with Armour Standard FSH 264-151X. Raacke *et al.* (7), applied zone electrophoresis to further purify preparations made by the method of Li *et al.* (1) and obtained an FSH with MED of 25 μg. Steelman and Segaloff (4, 5) used gradient elution from DEAE-cellulose to produce FSH with a potency 30 to 40 times the Armour Standard 264-151X. The preparation made by Ellis (14) by displacement elution from DEAE-cellulose had a potency 20 times that of Armour 264-151X; further purification by zone electrophoresis resulted in a potency of 20 to 40 times this Armour Standard. It is noteworthy that Fraenkel-Conrat *et al.* (3) prepared FSH with a MED of 2.5 to 3.0 μg. using only techniques of salt, solvent and isoelectric precipitation.

It has been noted that on administration of increasing dosages of the main FSH-containing fractions estrogen production (judged by growth and distension of the uterus) occurred at lower levels than were necessary for repair of interstitial tissue. If, as is often regarded established, ICSH must be present with FSH for the production of physiological amounts of estrogen, the results indicate that only one-tenth of the amount of ICSH is required for estrogen production as for repair of interstitial tissue. Inasmuch as accurate recognition of interstitial cell repair is somewhat difficult in the presence of high levels of FSH, it is noteworthy that dosages minimally capable of repairing interstitial tissue were approximately the same as those which doubled ventral prostate weight.

A definite improvement in contamination of sheep FSH with ICSH was achieved in fraction IV-19F, judged by the criteria of interstitial repair, uterine response, and prostatic enlargement. Previous investigators noted

[7] The primary gonadotrophic stimuli against which FSH was tested in normal female rats were chorionic gonadotrophin, pregnant mare serum gonadotrophin (PMS) or sheep pituitary FSH; in hypophysectomized rats, PMS or FSH.

uterine enlargement on the administration of from 3 to 5 times (3, 33) the minimal dose for follicle stimulation. Estrous distension was found by Fraenkel-Conrat *et al.* (3) at 5 times, and luteinization of follicles at 15 times, the MED for FSH. Interstitial repair was reported on administration of multiples varying from 10 times (6, 8) to 20 or 50 times (1, 3, 9) the MED for FSH. Although the most highly purified sheep FSH preparations of Ellis (14)[8] and of Steelman and Segaloff (4, 5) contained ICSH (LH), the data to permit comparison in terms of interstitial repair, prostate or uterine enlargement were not presented.

The problem of freeing FSH from ICSH effects does not appear to be unique to sheep FSH. No follicle stimulant of natural origin (pituitaries of any species, menopause or castrate serum or urine) is known to us which did not at some relatively low multiple of the minimal dose for follicle development also show uterine stimulation and luteinization or interstitial cell stimulation. Of the follicle stimulants of pituitary origin, the pig FSH of Greep *et al.* (34) appeared to be freest of such effects (inasmuch as no uterine growth was found at 25 times, or interstitial repair at 35 times the minimal dose for follicle development). However, repetition of the preparative procedure resulted in an FSH-containing fraction which caused interstitial cell repair at 20 times the FSH minimum (9).

It is possible that procedures involving proteolytic digestion may prove of value in further separation of ICSH from sheep FSH, inasmuch as ICSH has been shown to be less stable than FSH to the action of certain proteases (35–38). The most promising report of the utilization of this principle is that of Steelman *et al.* (5, 39), who produced pancreatin-digested pig FSH with potency 30 to 40 times the Armour Standard 264-151X. Although this preparation produced uterine growth at doses which caused 100 to 150% increase in ovary weights of hypophysectomized rats, it produced no growth of ventral prostate of hypophysectomized male rats receiving approximately 2000 times the minimal dose for FSH (250 μg. per day for 21 days).

As long as methods are available and being applied by which the biological purity of FSH can be improved, it is consistent to assume that the luteinization effects and stimulation of uterus, repair of interstitial tissue and growth of prostate observed at high doses are due to contamination with ICSH. At the present stage of purification of sheep FSH, it is premature to predict whether a certain amount of ICSH-like activity is an intrinsic property of the FSH molecule.

REFERENCES

1. Li, C. H., M. E. Simpson and H. M. Evans: *Science* **109**: 445. 1949.
2. Simpson, M. E., C. H. Li and H. M. Evans: *Endocrinology* **48**: 370. 1951.

[8] A preparation obtained by DEAE-cellulose chromatographic purification by Dr. Ellis and found by him to have a potency 10 times Armour 264-151X, was determined in this laboratory to have a MED for FSH of 15 μg. with ICSH at 10 times this MED.

3. Fraenkel-Conrat, H. L., M. E. Simpson and H. M. Evans: *Proc. Soc. Exper. Biol. and Med.* **45**: 627. 1940.
4. Steelman, S. L. and A. Segaloff: Abstracts, Endocrine Society 39th Meeting, N. Y., 1957. p. 104 (No. 158).
5. Steelman, S. L. and A. Segaloff: *Rec. Prog. Hormone Res.* **15**: 115. 1959.
6. Li, C. H. and K. O. Pedersen: *J. Gen. Physiol.* **35**: 629. 1952.
7. Raacke, I. D., A. J. Lostroh and C. H. Li: *Arch. Biochem. Biophys.* **77**: 138. 1958.
8. Jensen, H., M. E. Simpson, S. Tolksdorf and H. M. Evans: *Endocrinology* **25**: 57. 1939.
9. Van Dyke, H. B., S. Y. P'an and T. Shedlovsky: *Endocrinology* **46**: 563. 1950.
10. Fevold, H. L.: *Endocrinology* **24**: 435. 1939.
11. McShan, W. H. and R. K. Meyer: *J. Biol. Chem.* **135**: 473. 1940.
12. McShan, W. H., C. W. Kagawa and R. K. Meyer: *Proc. Soc. Exper. Biol. and Med.* **85**: 393. 1954.
13. Raacke, I. D. and C. H. Li: *In* Ion Exchangers in Organic and Biochemistry. Calmon, C. and T. R. E. Kressman, eds., Interscience, New York, 1957. p. 368.
14. Ellis, S.: *J. Biol. Chem.* **233**: 63. 1958.
15. Woods, M. C. and M. E. Simpson: *Fed. Proc.* **18**: 687. 1959.
16. Evans, H. M., M. E. Simpson, S. Tolksdorf and H. Jensen: *Endocrinology* **25**: 529. 1939.
17. Wooten, E., M. M. Nelson, M. E. Simpson and H. M. Evans: *Endocrinology* **63**: 860. 1958.
18. Gaddum, J. H.: *Pharmacol. Rev.* **5**: 87. 1953.
19. Greep, R. O., H. B. Van Dyke and B. F. Chow: *Proc. Soc. Exper. Biol. and Med.* **46**: 644. 1941.
20. Lostroh, A. J., P. G. Squire and C. H. Li: *Endocrinology* **62**: 833. 1958.
21. Squire, P. G. and C. H. Li: *J. Biol. Chem.* **234**: 520. 1959.
22. Evans, H. M., M. E. Simpson, W. Marx and E. Kibrick: *Endocrinology* **32**: 13. 1943.
23. Greenspan, F. S., C. H. Li, M. E. Simpson and H. M. Evans: *Endocrinology* **45**: 455. 1949.
24. Fraenkel-Conrat, J., H. Fraenkel-Conrat, M. E. Simpson and H. M. Evans: *J. Biol. Chem.* **135**: 199. 1940.
25. Simpson, M. E., H. M. Evans and C. H. Li: *Endocrinology* **33**: 261. 1943.
26. Lyons, W. R.: *Cold Spring Harbor Symposia on Quantitative Biology* **5**: 198. 1937.
27. Fraenkel-Conrat, H., M. E. Simpson, C. H. Li and H. M. Evans: *Anales Fac. de Med. de Montevideo* **25**: 169. 1940.
28. Ellis, S. and M. E. Simpson: *J. Biol. Chem.* **220**: 939. 1956.
29. Adams, E. and E. L. Smith: *J. Biol. Chem.* **191**: 651. 1951.
30. Fraenkel-Conrat, H. L., M. E. Simpson and H. M. Evans: *Anales Fac. de Med. de Montevideo* **25**: 159. 1940.
31. Steelman, S. L., A. Segaloff and R. N. Andersen: *Proc. Soc. Exper. Biol. and Med.* **101**: 452. 1959.
32. Steelman, S. L. and F. L. Pohley: *Endocrinology* **53**: 604. 1953.
33. Fevold, H. L.: *Endocrinology* **28**: 33. 1941.
34. Greep, R. O., H. B. Van Dyke and B. F. Chow: *Endocrinology* **30**: 635. 1942.
35. Evans, H. M., M. E. Simpson and P. R. Austin: *J. Exper. Med.* **58**: 545. 1933.
36. McShan, W. H. and R. K. Meyer: *J. Biol. Chem.* **126**: 361. 1938.
37. McShan, W. H. and R. K. Meyer: *Proc. Soc. Exper. Biol. and Med.* **40**: 699. 1939.
38. Chen, G. and H. B. Van Dyke: *Proc. Soc. Exper. Biol. and Med.* **40**: 172. 1939.
39. Steelman, S. L., T. L. Kelly, A. Segaloff and G. F. Weber: *Endocrinology* **59**: 256. 1956.

AN ASSAY FOR ANABOLIC STEROIDS EMPLOYING METABOLIC BALANCE IN THE MONKEY: THE ANABOLIC ACTIVITY OF FLUOXYMESTERONE[1] AND ITS 11-KETO ANALOGUE

J. C. STUCKI, A. D. FORBES, J. I. NORTHAM AND J. J. CLARK

Research Division of The Upjohn Company, Kalamazoo, Michigan

ABSTRACT

A procedure for conducting metabolic balance studies in the *Macaca mulatta* monkey was presented. A Latin square design employing four monkeys, four doses of fluoxymesterone and four drug periods, each preceded and succeeded by its own control period, produced linear response curves over the full dose range, 50–400 μg./kg./day, for nitrogen retention expressed as total N retained per day during drug periods, increased N retained during drug periods with respect to pre- and post-drug control periods and for body weight gains. Residual effects of treatment in any period on responses in the treatment period immediately following were negligible. Average estimates of increased N retained as a percentage of body weight gain compared favorably with the reported N content of mammalian muscle. A statistically balanced twin cross-over assay was studied. Fluoxymesterone was employed as the standard and 9α-fluoro-11-keto-17α-methyltestosterone as the test compound. Nitrogen retention, potassium retention and body weight gain demonstrated the anabolic potency of the test compound to be equal to that of fluoxymesterone. The efficiency of the experimental design was calculated to be 1.5 to 2. It is concluded that metabolic balance procedures can be employed for quantitative bioassay of anabolic agents in the monkey and probably in other species provided proper experimental design is used.

IT WAS first demonstrated by Kochakian and Murlin (1–3) in the castrate dog and later by Kenyon and co-workers (4, 5) in the human that androgens produce positive nitrogen balance and somatic growth. Nitrogen balance procedures are, however, time consuming and expensive. Consequently, though there are many reports on the relative anabolic activities of various androgens, these reports contain a minimum of data and lack statistical justification of the experimental design and the expressions of reliability and accuracy limits ordinarily required for acceptable bioassay.

The purpose of this communication is to present the description and statistical validation of a method for the quantitative bioassay of anabolic agents using metabolic balance in the monkey (*Macaca mulatta*) and to report the anabolic activity of two potent steroids, fluoxymesterone and 9α-fluoro-11-keto-17α-methyltestosterone (U-6596).

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[1] Halotestin®, The Upjohn Company, Kalamazoo, Mich.

METHODS AND MATERIALS

The balance ward

A large (approximately 20′×20′), locked, windowless room, air conditioned and humidity controlled (78° F, 50% relative humidity), was used for housing the monkeys. A timing device switched artificial lights on 12 hours and off 12 hours each day. Music was played over a loud speaker continuously during the light hours and served the purpose of eliminating extraneous noises. Admittance to the ward was strictly limited to the technician operating the ward, the consultant pathologist and the investigator in charge. The technician operating the ward worked seven days a week and conducted his activities in and about the ward so that no difference between week days and week ends was apparent to the animals. The daily schedule of activities was kept constant both in respect to order of operations in the ward and time at which particular operations were conducted. It is our opinion that a constant and predictable ward environment is essential if reliable estimates of anabolic activities are to be obtained.

Cages and animal handling

The cages (Fig. 1) were designed in this laboratory and fabricated from galvanized steel by The Kalamazoo Sheet Metal Mfg. Co., Kalamazoo, Michigan. Pans and collection funnels were made of stainless steel. Each unit consisted of two cages, one mounted on top of the other. All accessories were interchangeable. Either large mesh bottoms and trays for animal storage or small mesh bottoms and funnels for metabolism studies were available. Upper side walls and tops were originally wide mesh expanded steel but experience showed that a smaller mesh was necessary to keep the animals from throwing food and excreta from the cage. The illustrated cages have had outer small mesh screens affixed to them to serve this purpose.

The animals were kept in the cages without chains or collars and were ordinarily moved from cage to cage by means of a "catching box" hung on the front of the door. A sliding door on the cage and one on the box lifted together allowed the animal to go from the cage to the box and vice versa. The monkeys were easily trained to go into or out of the box as desired. The catching box was tared to permit accurate determination of body weight. The sliding door was secured with a snap lock (not shown).

The food box, fitted with a snap cover, was attached to the sliding panel of the door so that the hand hole in the panel and one in the box were opposed. Any food removed from the box by the monkey could only be taken into the cage. The watering device consisted of a bottle and a curved stainless steel tube in a rubber stopper. The bottle was fitted into the bottle rack and secured by means of a steel rod and a second rubber stopper. About $1\frac{1}{2}$ inches of the stainless tube protruded into the cage. A double rack allowed the feeding of liquid diets if desired. The animals obtained water by sucking on the end of the tube. When first caged, animals often did not know how to drink from this device, but were easily taught to drink if orange juice was used instead of water and if banana pulp was smeared on the end of the tube.

Metabolic collection flasks containing H_2SO_4 and thymol were positioned under the collection funnel and held in place with a square of plywood. The top of the lower cage was partly constructed of solid material so that when the animal was placed in the lower cage it could not reach through the screen and disturb the flask prior to collection of the sample.

Animals

The experimental animal was the ovariectomized young female *Macaca mulatta* on limited food intake. The animals were obtained from an importer and carefully conditioned after receipt. During the conditioning period they were isolated, tuberculin

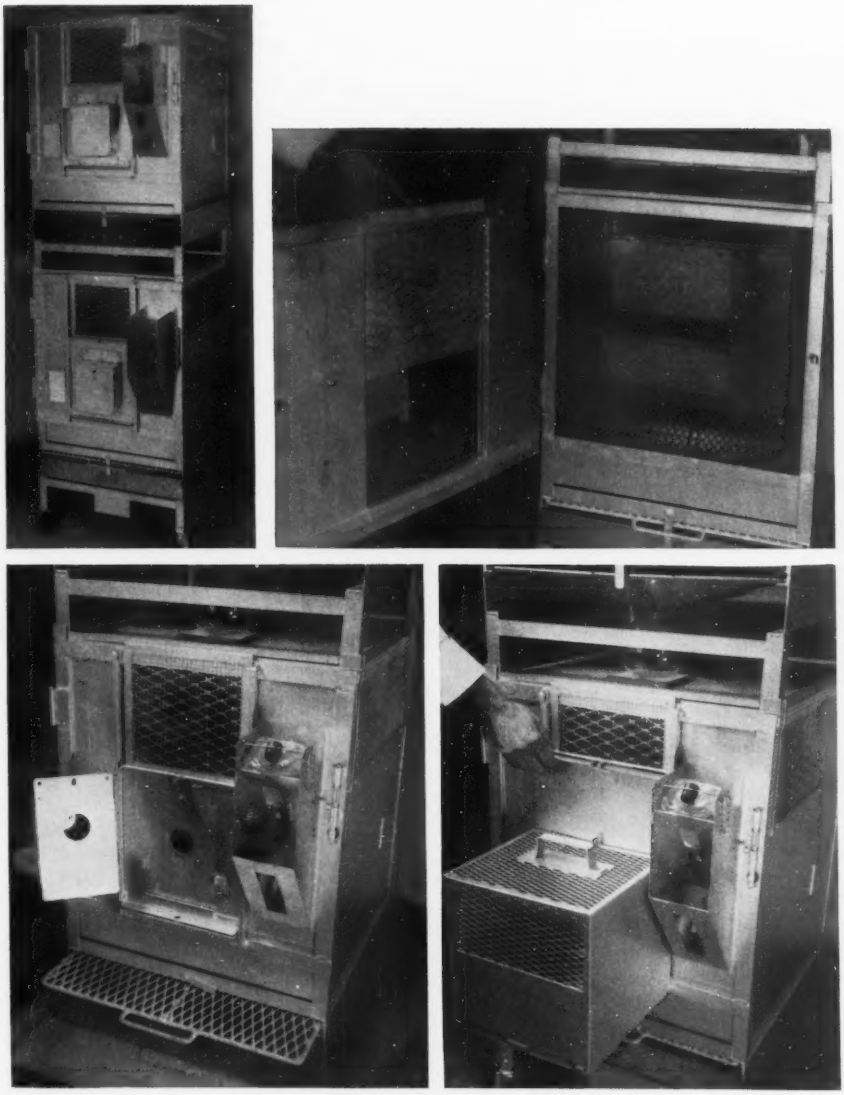


FIG. 1. Details of construction and use of monkey metabolism cage.

tested, given a prophylactic course of tetracycline therapy orally and wormed with N-butyl chloride and castor oil if fecal samples demonstrated this to be necessary. The butyl chloride did not eliminate hookworms entirely from the monkey but did reduce drastically the severity of the infections. Reinfection was not a problem under conditions of ward operation.

The animals were ovariectomized under pentobarbital anesthesia employing full

sterile technique. Balance studies were begun 3 or 4 months after ovariectomy and only after the animals were fully acclimated to the balance ward. Periodically, between balance studies, the animals were tuberculin tested.

Food, feeding and drug administration

A single constant composition lot of Purina monkey chow was obtained from the Ralston Purina Company, St. Louis, Mo., and stored at −10° C until a day or two prior to use. The animals were fed a measured amount of food once a day at about 7:00 A.M. The amount was less than the animal would eat if fed *ad libitum*, but was sufficient to allow a very slow rate of weight gain when no anabolic agent was administered. With rare exceptions, the food ration was consumed within an hour or two.

Multiple vitamin concentrate (Zymadrops—Upjohn) was fed twice daily to insure adequate vitamin intake. Each morning a tray containing two cubes of sucrose for each monkey was carried into the ward. One cube was soaked with 0.3 ml. Zymadrops and fed immediately before the chow and the second vitamin soaked cube was fed at 2:30 P.M. Oral administration of test steroids was facilitated by the fact that the monkeys eagerly awaited the vitamin feedings. The steroids in alcohol were placed on the sugar cubes the day before the cubes were fed. The alcohol had evaporated by the time the cubes were fed. Since the steroids were put into the cubes in another room and the cubes were carried into the ward each morning during control and drug periods alike, and since the vitamins masked any taste the steroid might have had, it was not necessary to employ placebo controls when conducting anabolic steroid assays. The steroids were administered on a μg./kg. of body weight basis twice a day.

The animals were given 200 ml. of deionized water to drink twice a day at the time the vitamin concentrates were administered. The water was measured not to determine consumption, but in order that there be no excess water to complicate collection and handling of the metabolic samples.

Balance technique

All animals were kept on balance continuously. Metabolic collections were made and body weights were determined for each animal every other day. Collections and weights on ½ of the animals in the ward were taken on one day and ½ on the following day. The daily schedule of ward operation is summarized as follows: in the morning clean cages were prepared for occupany and ½ of the animals were weighed and moved to the clean cages. All animals were given the vitamin impregnated sugar cubes, water and food rations. Following the feeding, metabolic samples were collected from the vacated cages using deionized water, brushes and scrapers to quantitatively collect all urine, feces and residual food (uneaten, uncontaminated food was removed, weighed and subtracted from that fed in making balance calculations). In the afternoon the ward was prepared for the following day's operations and the animals were again given the sugar cubes and water.

After being removed to the laboratory, the metabolic collections were homogenized in a Waring blendor and diluted to constant volume. An aliquot was ground even finer in a glass homogenizer. After further dilution, duplicate aliquots of the fine homogenate were taken for analysis by a modified micro-Kjeldahl nitrogen method. A $CuSO_4$-K_2SO_4 catalyst in concentrated H_2SO_4 was employed for an 18-hour digestion. The digests were distilled into boric acid solution and the distillates titrated with 0.02N H_2SO_4, using Tashiro's indicator. In some experiments fine homogenate was centrifuged and the K content of the supernate was measured with a flame photometer. Elemental balances were calculated in mg./day by comparing the amount of the element fed with that recovered in the metabolic collections.

RESULTS AND DISCUSSION

Latin square design

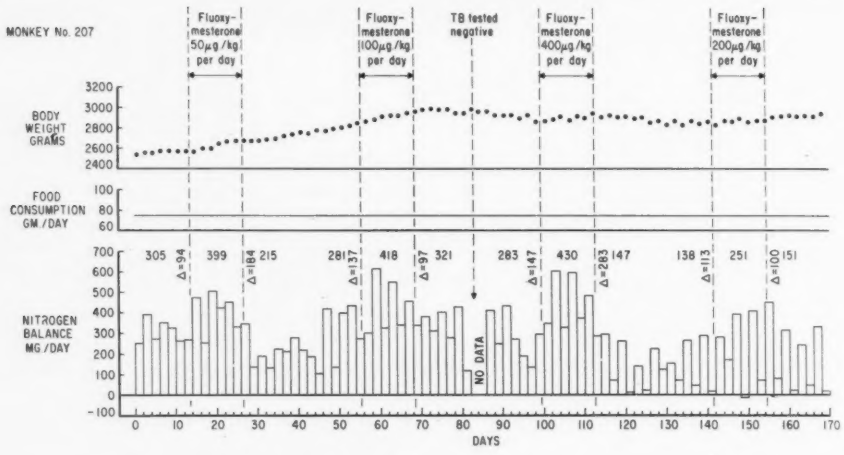
A Latin square design was employed initially to gain information on the number of monkeys, trials and doses required for adequate determination of potency ratios, and to allow proposal of an assay procedure. Fluoxymesterone (9α-fluoro-11β-hydroxy-17α-methyltestosterone was employed in this experiment. This steroid has been reported to be an extremely potent myotrophic agent as well as an androgen in rats (6, 7) and has been shown to be a very effective anabolic agent in man (8–10). The drug was administered orally twice daily in equally divided doses to four monkeys according to the Latin square pattern evident from examination of Figure 2.

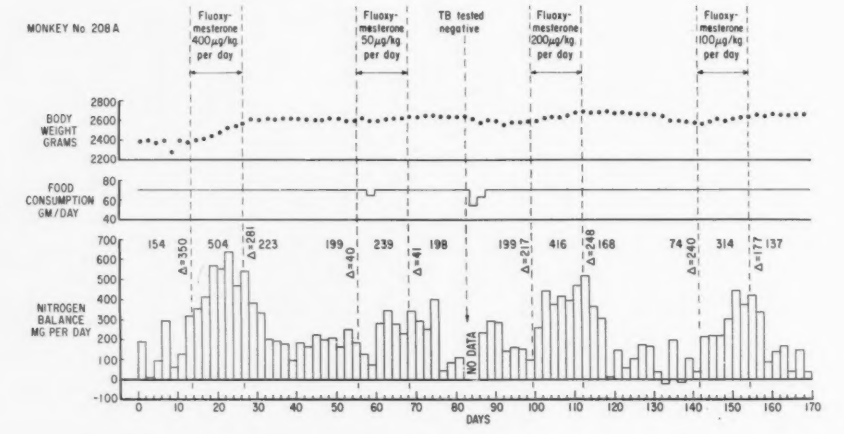
Dose periods were fourteen days in length and each was preceded and succeeded by its own 14 day control period. (Tuberculin testing halfway through the experiment resulted in the loss of 4 days' data, so that the post-drug control period following the second drug period for each animal was only 12 days in length.) The entire experiment covered a period of 24 weeks. The particular Latin square employed is one that permits, among other things, convenient estimation of treatment means adjusted for residual effects of treatments applied in the immediately preceding period (11).

Results obtained on each animal appear graphically in Figure 2. The following measurements were employed to evaluate the responses produced by the administration of the hormone: 1) average nitrogen (N) retained per day during the 14 day drug period, 2) average N retained per day during the 14 day pre-drug control period, 3) average N retained per day during the 14 day post-drug control period, 4) the differences between average daily retention during drug periods and control periods (Δ from pre and Δ from post), and 5) body weight gain during drug periods. Except when otherwise specified, all of the seven individual balance values obtained during each period were employed in computing average N retained. Body weight gains were computed by subtracting the last weight prior to drug administration from the first weight taken after drug administration was stopped. The data are summarized in Tables 1–3 and in Figures 3–7. Appropriate statistical analyses of these data allowed the following conclusions to be made:

Nitrogen retention

The balanced Latin square design gave good linear dose-response curves for N retention (Figs. 3–5). The standard error of each dose mean is approximately 40 mg. N retained per day. The actual observed N retained during drug administration (Fig. 3) and the increased N retained with respect to pre-drug control periods (Fig. 4) and post-drug control periods (Fig. 5)


MONKEY No. 207
Fluoxy-mesterone 50μg/kg per day
Fluoxy-mesterone 100μg/kg per day
TB tested negative
Fluoxy-mesterone 400μg/kg per day
Fluoxy-mesterone 200μg/kg per day
BODY WEIGHT GRAMS
FOOD CONSUMPTION GM/DAY
NITROGEN BALANCE MG/DAY
305 Δ=94 399 Δ=184 215 281 Δ=137 418 Δ=97 321 283 Δ=147 430 Δ=283 147 138 Δ=113 251 Δ=100 151
NO DATA
DAYS


MONKEY No. 208A
Fluoxy-mesterone 400μg/kg per day
Fluoxy-mesterone 50μg/kg per day
TB tested negative
Fluoxy-mesterone 200μg/kg per day
Fluoxy-mesterone 100μg/kg per day
BODY WEIGHT GRAMS
FOOD CONSUMPTION GM/DAY
NITROGEN BALANCE MG PER DAY
154 Δ=350 504 Δ=281 223 199 Δ=40 239 Δ=41 198 199 Δ=217 416 Δ=248 168 74 Δ=240 314 Δ=177 137
NO DATA
DAYS

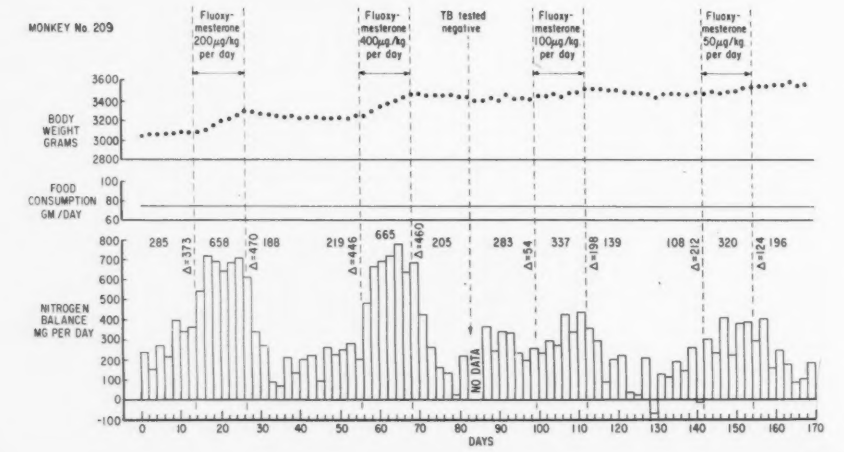

MONKEY No. 209
Fluoxy-mesterone 200μg/kg per day
Fluoxy-mesterone 400μg/kg per day
TB tested negative
Fluoxy-mesterone 100μg/kg per day
Fluoxy-mesterone 50μg/kg per day
BODY WEIGHT GRAMS
FOOD CONSUMPTION GM/DAY
NITROGEN BALANCE MG PER DAY
285 Δ=373 658 Δ=470 188 219 Δ=446 665 Δ=460 205 283 Δ=54 337 Δ=198 139 108 Δ=212 320 Δ=124 196
NO DATA
DAYS

TABLE 1. SUMMARY AVERAGES OF N RETAINED IN LATIN SQUARE DESIGN ACCORDING TO DOSE, MONKEY AND PERIOD

Dose fluoxymesterone μg/kg./day	Average mg. N retained/day				
	Pre-Control	Δ-Pre	Drug Period	Δ-Post	Post-Control
50	188	101 (110)*	289 (309)	97 (107)	192
100	202	139 (139)	341 (338)	135 (128)	206
200	209	235 (250)	444 (449)	246 (258)	198
400	215	313 (289)	528 (506)	330 (315)	198
Monkey No.					
207	252	122	374	166	208
208A	156	212	368	186	182
209	224	271	495	313	182
210A	182	182	364	142	222
Period No.					
1	228	236	464	250	214
2	228	215	443	191	252
3	227	119	346	193	153
4	131	219	350	175	175

* Figures in parentheses are values adjusted for residual effects from previous dosing period.

appear to be equally reliable for determination of the relative potency of two compounds. As will be seen later, the Δ from pre-drug control values may be preferable, however, because they can be readily translated into observed weight gains during dosing.

Analysis of the possible residual effects of treatment in any period on response in the treatment period immediately following does not reveal any statistically significant residual effects throughout the entire experiment. Such adjustments as can be made for possible residual effects are of little practical significance (Table 1, Figs. 3–5).

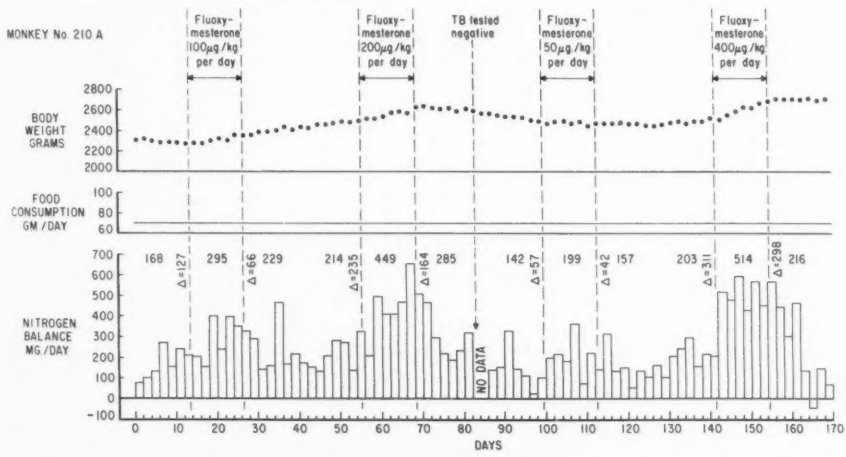


FIG. 2. Balance charts for ovariectomized female monkeys administered various doses of fluoxymesterone. Average mg. N retained during each period and Δ's from control periods appear above N balance bars.

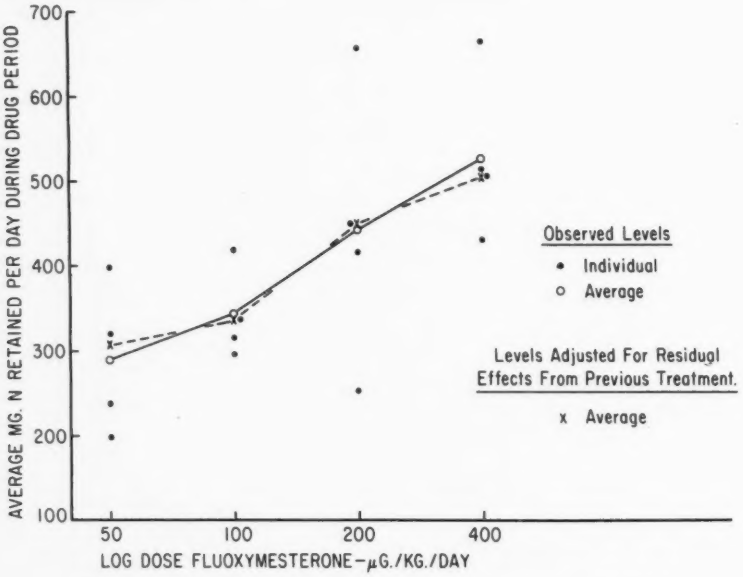


FIG. 3. Effect of fluoxymesterone in ovariectomized monkeys on average N retention during drug periods.

TABLE 2. WEIGHT GAINS (IN GM.) DURING DOSING ACCORDING TO DOSE, MONKEY, AND PERIOD

Monkey no.	1	2	3	4	Monkey averages
207	A + 93	B +115	D + 85	C + 11	+ 76
208A	D +185	A + 41	C +103	B + 69	+100
209	C +199	D +213	B +106	A + 47	+141
210A	B + 85	C +134	A − 15	D +164	+ 92
Period avg.	+142	+126	+ 70	+ 73	

TABLE 3. ESTIMATED NITROGEN PERCENTAGE OF WET MUSCLE WEIGHT ACCORDING TO MONKEY, PERIOD, AND DOSE LEVEL

Monkey no.	1	2	3	4	Monkey averages
207	A 1.4	B 1.7	D 2.4	C 14.4	4.97
208A	D 2.6	A 1.4	C 2.9	B 4.9	2.95
209	C 2.6	D 2.9	B .7	A 6.3	3.12
210A	B 2.1	C 2.5	A *	D 2.7	2.43
Period Avg.	2.17	2.12	2.00	7.07	

* No estimate made on account of weight loss.

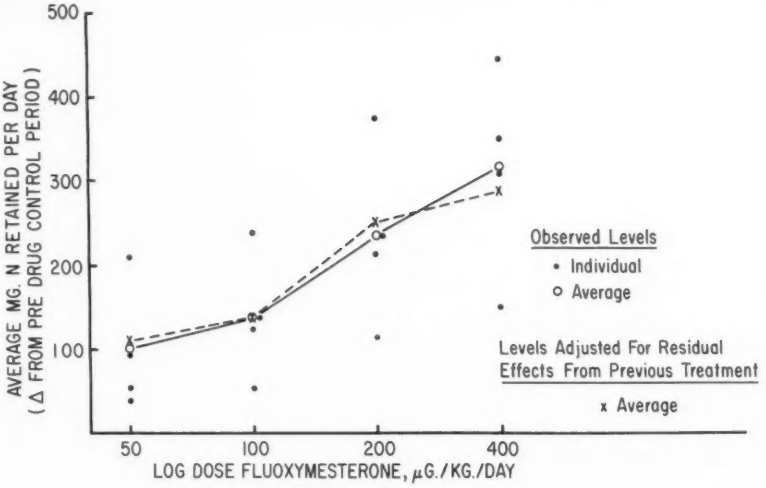


FIG. 4. Effect of fluoxymesterone in ovariectomized monkeys on changes in N retention from pre-drug control periods.

A good consistency is seen when N retentions during drug and control periods are averaged according to dose (Table 1). When the same averages are computed according to monkeys, however, considerable variation is in evidence (Table 1). When averages are made according to periods, it can be seen that initially good consistency was in evidence but a sustained drop of about 100 mg. N retained per day occurred during the third drug period

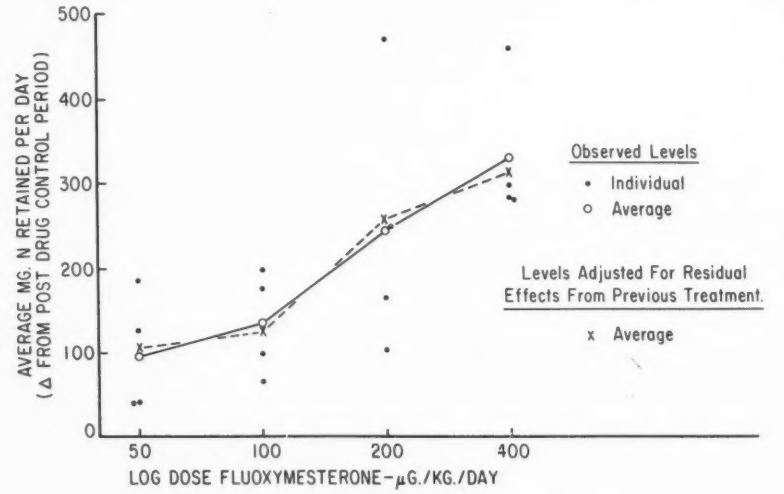


FIG. 5. Effect of fluoxymesterone in ovariectomized monkeys on changes in N retention from post-drug control periods.

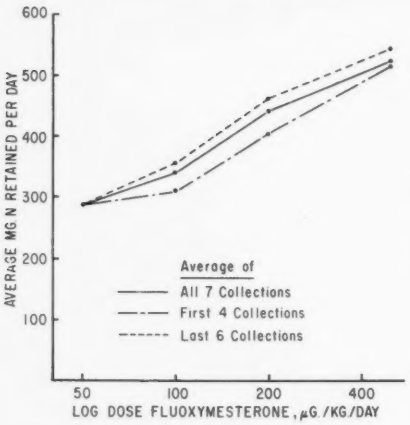


FIG. 6. Expression of the effect of fluoxymesterone in ovariectomized monkeys on average N retention during drug periods using a varying number of metabolic sample collections.

(Table 1). These observations, particularly the last, suggest that a statistically balanced design must be employed in the assay of anabolic steroids by metabolic balance methods to eliminate the effect of such variation.

The fact that post-drug control period averages do not differ markedly from pre-drug control period averages suggests that it may be possible to drastically reduce the length of the control periods between drug periods.

Examination of dose-response curves (Fig. 6) based on various numbers of collections in the drug period suggests that, over the dose range 100–400 μg./kg./day, a reduced number of measurements may give an adequate

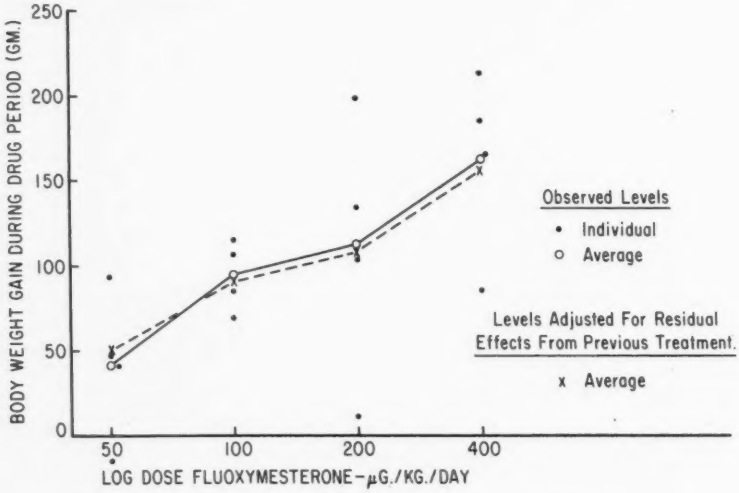


FIG. 7. Effect of fluoxymesterone in ovariectomized monkeys on body weight gain during drug periods.

dose response curve for the determination of relative potency. Furthermore, the first measurement of N retention after drug administration appears to be lower on the average than subsequent measurements, and might be profitably eliminated from the calculations.

Weight gains

A linear dose-response curve was also obtained using body weight gain as the measurement of anabolic response (Fig. 7). Again, there were no appreciable residual effects from previous dosing. On the average, an increase of 1 gm. body weight in 2 weeks is associated with an increase (Δ from pre control) of 2 mg. N retained per day. On the assumption that all weight gains are due to accretion of muscle, the relationship suggests that 2.8% of the wet muscle weight is N. This corresponds closely with the actual N content (2.7–3.5%) of muscles of various mammalian species (12).

Examination of estimates of extra nitrogen (Δ pre) retained as percentages of wet muscle weight (body weight gain) for individual animals (Table 3) shows that there is considerable variation. Deviant estimates appear to be particularly associated with periods 3 and 4 and/or with the lower drug doses. In spite of this variation, the average agreement between calculated values and reported N content of muscle (12) gives assurance that actual protein anabolism occurred under the stimulation of the anabolic agent.

It should be noted that there was a virtual absence of weight gain during control periods despite relatively constant positive N balance. The positive

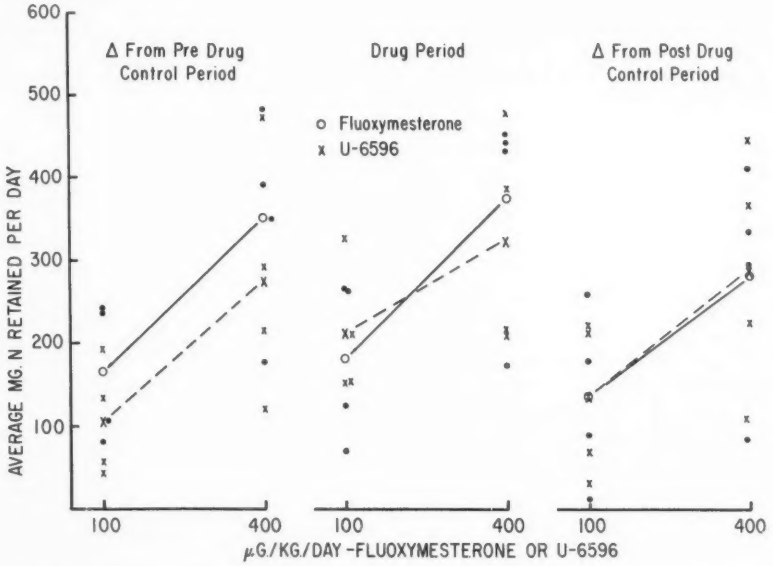


FIG. 8. Comparison of effects of fluoxymesterone and U-6596 on N retention.

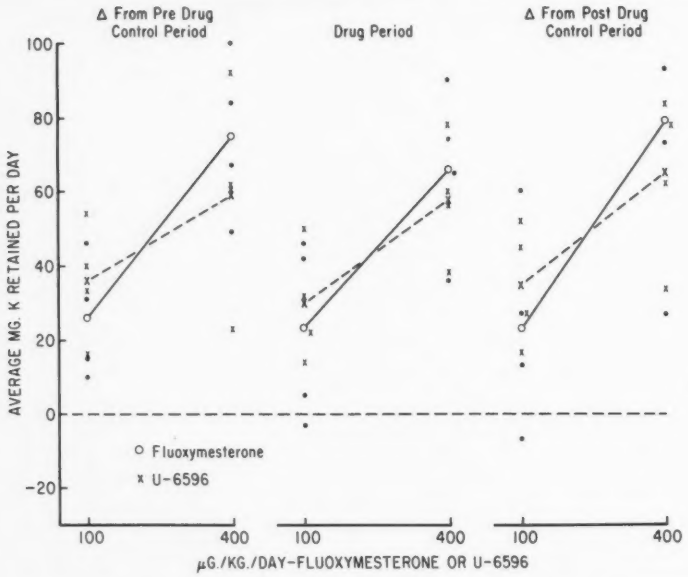


FIG. 9. Comparison of effects of fluoxymesterone and U-6596 on K retention.

N balances observed during these periods and a portion of the positive N balance seen during periods of weight gain were probably due mostly to a relatively constant failure to recover all excreted N. Such error would be reflected in positive N balance. For this reason also, it is felt that *increases* in N retention after drug administration (Δ pre) are more meaningful than total N retentions.

Twin crossover assay of anabolic steroids

After the completion of the Latin square experiment, it became possible to study quantitative assays of anabolic agents. The twin crossover assay (13) was chosen because it is thought to represent the simplest type of de-

TABLE 4. POTENCY OF U-6596, RELATIVE TO FLUOXYMESTERONE BY DIFFERENT METHODS OF ESTIMATION

Data used	Potency of U-6596 (×fluoxymesterone)	95% Confidence limits for potency	Standard error per assay (%)
N—Δ pre	.59	.03–1.98	38
N—on dose	.90	.14–4.13	38
N—Δ post	1.04	.22–5.31	37
K—Δ pre	.90	.37–2.01	26
K—on dose	.98	.55–1.73	19
K—Δ post	.96	.44–2.04	24
Body wt. gain on dose	.79	.40–1.41	20

sign that meets the minimal requirements for a satisfactory parallel line assay. Two doses of the standard, two doses of the unknown, 8 monkeys and 2 drug periods were employed. Fluoxymesterone was employed as the standard and its 11-keto analogue (9α-fluoro-11-keto-17α-methyltestosterone or U-6596) was used as the test or unknown steroid. U-6596 is biologically similar in rats to fluoxymesterone (6, 7). Each drug period was reduced to 10 days (5 metabolic sample collections). The first period was preceded by a 10 day control period, a 10 day control period separated the two drug periods, and a 10 day control period followed the second drug period. The last four collections only in each period were employed in the analysis of the data. In addition to N balance and body weight gain, potassium (K) balances were also determined and used for the estimation of anabolic potency.

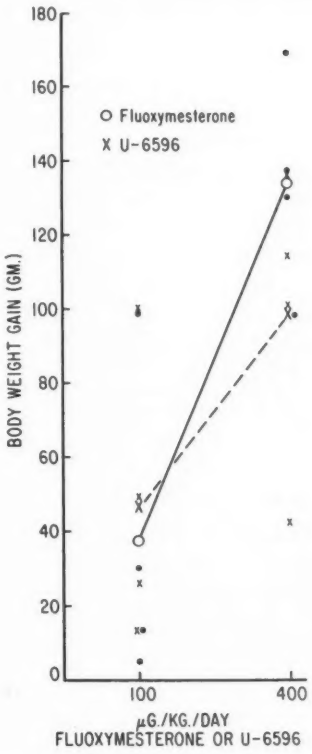


FIG. 10. Comparison of effects of fluoxymesterone and U-6596 on body weight gain.

Results appear in Figures 8–10. The various estimates of potency of U-6596, relative to fluoxymesterone were made by methods described by Finney (13) and appear in Table 4. It can be seen that each of the seven estimates of relative potency lies within the 95% confidence limits for relative potency determined for each of the other estimates. The estimates of relative potency average slightly less than 1 but all 95% confidence limits include the value 1. It is concluded that U-6596 has an anabolic potency approximately equal to that of fluoxymesterone.

Computation of the per cent standard error per assay indicates that K balance and body weight gain data gave better precision than did the N balance data in this experiment. The coefficient of variation (intra-monkey standard deviation as a percentage of the mean) for all three variables, N, K, and body weight gain during drug administration is approximately 30%.

An estimate of the gain from the use of this twin crossover design can be made from a comparison of the measures of inter-monkey and intra-monkey variation which the design provides. Each of these sources of variation is associated with only 4 degrees of freedom (when 8 monkeys are dosed on

2 different occasions), hence, further experience with the twin crossover design will be necessary to evaluate the design properly. It appears, however, from the analysis of this single experiment that the efficiency of the design for K and weight gain data was approximately 2, i.e., 8 monkeys each tested on two dose periods lead to potency estimates at least as good as if 32 animals had been tested on one dose period each. For N data, the efficiency was approximately 1.5.

Clearly, metabolic balance procedures can be employed for quantitative bioassay of anabolic agents in the ovariectomized monkey provided proper experimental design is used. Since species differences in ability to show dose related responses to anabolic steroids would not be expected, the use of similar or identical design seems warranted for assays in other species, including man.

Whether or not the relative potencies of anabolic steroids in man are predictable from monkey data remains to be seen. While studies designed to resolve this problem are in process and will be reported at a future time, it should be noted from the present study that the monkey showed a marked and graded response to fluoxymesterone in a dose range that is comparable to that (on a mg./kg. basis) which produces pronounced anabolism in man (8–10).

Acknowledgment

The authors are grateful to Mrs. Ruth Conant, Mrs. Ruta Kupsis, Mr. George Lund, Mr. Harold Albert and Mr. Ernest Markovich of The Upjohn Company for valuable technical assistance and to Prof. T. C. Ruch, Washington University School of Medicine and Dr. Ernest Knobil, Harvard Medical School for advice and encouragement.

REFERENCES

1. Kochakian, C. D. and J. R. Murlin: *J. Nutrition* **10:** 437. 1935.
2. Kochakian, C. D. and J. R. Murlin: *Am. J. Physiol.* **117:** 642. 1936.
3. Kochakian, C. D.: *Endocrinology* **21:** 750. 1937.
4. Kenyon, A. T., K. Knowlton and I. Sandiford: *Ann. Internal Med.* **20:** 632. 1944.
5. Kenyon, A. T., I. Sandiford, A. H. Bryan, K. Knowlton and F. C. Koch: *Endocrinology* **23:** 135. 1938.
6. Herr, M. E., J. A. Hogg and R. H. Levin: *J. Am. Chem. Soc.* **78:** 500. 1956.
7. Lyster, S. C., G. H. Lund and R. O. Stafford: *Endocrinology* **58:** 781. 1956.
8. Bartter, F. C.: *Am. J. Med.* **22:** 797. 1957.
9. Kennedy, B. J.: *Cancer* **10:** 813. 1957.
10. McGavack, T. H. and W. Seegers: *Am. J. Med. Sci.* **235:** 125. 1958.
11. Cochran, W. G. and G. M. Cox: *Experimental Designs*, 2nd ed. 1957, pp. 133–138, John Wiley and Sons.
12. Spector, W. S.: *Handbook of Biological Data*, 1956, p. 72, Saunders.
13. Finney, D. J.: *Statistical Methods in Biological Assay*, 1952, pp. 266–273, Hafner.

ON THE MOVEMENT OF PIGMENT GRANULES IN FROG MELANOCYTES[1]

M. RUTH WRIGHT AND AARON B. LERNER

Section of Dermatology, Department of Medicine, Yale University School of Medicine, New Haven, Connecticut

ABSTRACT

MSH, caffeine and some physical factors induced dispersion of melanin granules in the melanocytes of the frog, *Rana pipiens*. Melatonin, noradrenaline and hydrocortisone reversed dispersion produced by many agents. Acetylcholine reversed only the dispersion caused by MSH. In sucrose solution containing normal amounts of calcium and potassium, caffeine was inhibited in part; MSH, melatonin, noradrenaline and hydrocortisone were inhibited completely. Caffeine could utilize lithium ions for the dispersion process, and melatonin and noradrenaline reversed this dispersion.

It is suggested that melanin granule movement is produced by cytoplasmic streaming associated with ionic exchange between intra- and extracellular media and that substances affecting melanin dispersion, directly or indirectly produce a change in the specific ionic permeability of the cell membrane.

IN THIS paper we will report experiments carried out with five factors, enumerated below, that cause dispersion of pigment cell granules in frog melanocytes.

1. Melanocyte stimulating hormone (MSH)
2. Caffeine
3. Low osmotic activity
4. Low pH
5. Hypotonic sucrose solution

The ability of melatonin, noradrenaline, acetylcholine and hydrocortisone to reverse dispersion produced by these factors was also studied.

EXPERIMENTAL

Preparation of Frog Skin

Skin from the frog *Rana pipiens* was prepared and mounted on rings as described previously (1). Four samples were obtained from each frog—two from the legs and two from the thighs. For each experiment four samples were taken from each of eight frogs and arranged in four groups as follows:

Group A	LL1	LT2	RT3	RL4	LL5	LT6	RT7	RL8
Group B	RL1	LL2	LT3	RT4	RL5	LL6	LT7	RT8
Group C	RT1	RL2	LL3	LT4	RT5	RL6	LL7	LT8
Group D	LT1	RT2	RL3	LL4	LT5	RT6	RL7	LL8

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The first letter L and R refers to the left or right extremity of the frog. The second letter L or T refers to leg or thigh; and the number 1, 2, 3, 4, 5, 6, 7 or 8 refers to the frog used. Thus LL1 means skin from the left leg of frog 1. RT4 indicates skin from the right thigh of frog 4, etc. This arrangement gave four groups that could be tested simultaneously and which were as comparable as possible (2). It will be noted that each group contained one skin specimen from each frog. Because eight frogs were used, there were two left legs, right legs, left thighs and right thighs in each group.

All skin specimens were soaked in four changes of Ringer's solution over a two hour period before any observations were made.

Changes in the state of dispersion of melanin granules within the melanocyte were measured as changes in reflectance when the whole skin specimen was placed over a search unit attached to a photoelectric meter (1). All readings were taken with the skin immersed in 20 ml. of solution in a 50 ml. beaker. The original reading of the skin after soaking in Ringer's solution for two hours was taken as its individual base line, and an increase or decrease in reflectance was measured from this. The 32 specimens in the experimental plan could be read over a ten minute period so that by taking readings continuously it was possible to follow the change in each individual specimen at ten minute intervals. The graphs were prepared by totaling the changes in reflectance within each treatment group and plotting these values against time.

Solutions Used

Ringer's solution. 6.5 g. NaCl, 140 mg. KCl, 120 mg. $CaCl_2$ and 200 mg. $NaHCO_3$ were dissolved in distilled water and the solution was made up to a volume of 1 liter. The pH was 7.3. When a hypotonic medium was needed, 10 ml. of Ringer's solution was removed from the 50 ml. beaker and replaced with 10 ml. of distilled water. When it was necessary to increase the hydrogen ion concentration, the pH was lowered to 5.6 by adding 0.5 ml. 0.1 N hydrochloric acid to each beaker containing 20 ml. of Ringer's mixture.

Melanocyte stimulating hormone (MSH). Beef posterior pituitary powder from the Armour Laboratories was extracted with water and lyophilized as described previously (1). One mg. of the lyophilized product was dissolved in 1 ml. of Ringer's solution. 0.2 ml. of this mixture was diluted to 100 ml. with Ringer's solution, and 7.5 ml. of the new mixture was diluted to 10.0 ml. 0.2 ml. of the final product containing 7.5 units of MSH was then added to the 20 ml. of Ringer's solution in which the skin was immersed.

Caffeine. 2.9 gm. caffeine was dissolved in Ringer's solution and was made up to a volume of 1 liter. Seven ml. of Ringer's mixture was removed from each beaker and replaced by 7 ml. of caffeine solution so that the final concentration was 1 mg. caffeine per ml.

Melatonin. Two gm. of lyophilized, defatted, beef pineal powder were extracted for melatonin (3, 4). The final product was dissolved in 100 ml. Ringer's solution. 0.3 ml. of this mixture was diluted to 10 ml. with Ringer's, and 0.2 ml. of this diluted solution was addded to each beaker.

Noradrenaline. 1.0 mg. of noradrenaline bitartrate was dissolved in 10 ml. Ringer's solution. 0.2 ml. of this preparation, unless otherwise specified, was added to each beaker.

Acetylcholine. 1.0 mg. of acetylcholine bromide was dissolved in 10 ml. Ringer's solution. 0.2 ml. of this mixture, unless otherwise specified, was added to each beaker. Experiments carried out with solutions containing equimolar quantities of sodium bromide showed that the bromide ion had no effect on the melanocytes.

Hydrocortisone. The sodium salt of hydrocortisone hemisuccinate containing 100 mg. of hydrocortisone was dissolved in 5 ml. Ringer's solution. 0.2 ml. of this mixture was added to each beaker.

Sucrose. The sucrose solution contained 78.7 gm. of sucrose, 140 mg. KCl and 165 mg. $CaCl_2 \cdot 2H_2O$ per liter.

Lithium chloride. 4.74 g. LiCl, 140 mg. KCl and 165 mg. $CaCl_2 \cdot 2H_2O$ were dissolved in water and made up to a volume of 1 liter.

RESULTS

When skin was placed in Ringer's solution containing MSH, darkening occurred which resulted in decreased reflectance. Dispersion of melanin granules towards the periphery of the cell could be seen microscopically. Maximum dispersion occurred in about 50 minutes. Thereafter only a slight increase in darkening occurred. Melatonin, noradrenaline and hydrocortisone all produced rapid and marked reversal of the darkening reaction (Fig. 1). The effect of hydrocortisone was not well maintained. Acetylcholine produced marked reversal in about 50% of the skin specimens. This was in contrast to the action of the other three aggregating substances which induced reversal in all skin samples but to a varying extent. When maximum doses of melatonin, noradrenaline and hydrocortisone were used, the same amount of lightening resulted with each agent (Fig. 2). In no case was complete reversal of dispersion produced. The cells did not return to their initial state. The maximum dose of the lightening agent was determined by finding the point at which a further increase in concentration did not produce an increase in lightening.

Immersion of skin in caffeine solution produced dispersion of melanin

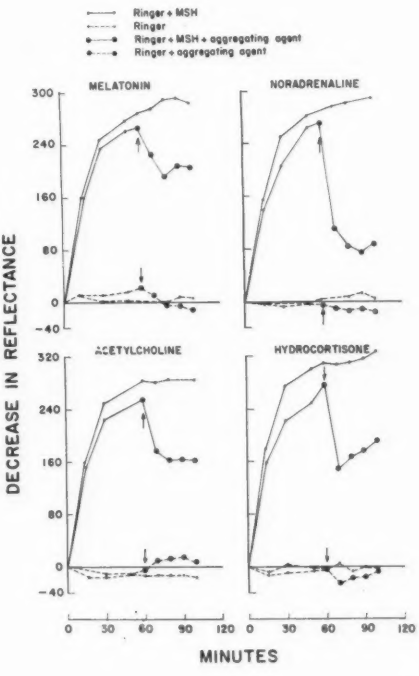


FIG. 1. Frog skin specimens in Ringer's solution were darkened with MSH. A decrease in reflectance occurred with time. After 60 minutes the aggregating agent melatonin, noradrenaline, acetylcholine or hydrocortisone was added. All produced lightening of the skin. Control specimens treated with MSH alone continued to darken slightly. The control specimens in Ringer's solution showed little change and those with melatonin, noradrenaline or hydrocortisone lightened slightly. Acetylcholine in Ringer's solution produced slight darkening.

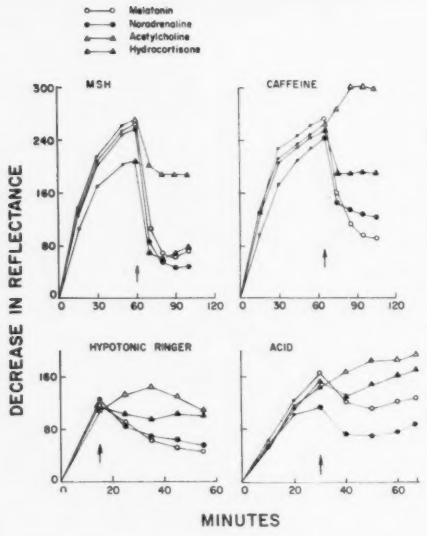


FIG. 2. Four comparable groups of skins were darkened with MSH, four with caffeine, four with hypotonic Ringer's solution and four with acidified Ringer's solution. At the time indicated by the arrow one group of specimens of each four was then treated with melatonin, one with noradrenaline, one with acetylcholine and one with hydrocortisone. All doses of the lightening agents were maximal. Acetylcholine reversed the action of MSH only. The other three agents reversed all four darkening mechanisms but to different degrees. In no case did complete reversal of darkening occur.

granules indistinguishable from that achieved by MSH. This dispersion was reversed readily by melatonin, noradrenaline and hydrocortisone (Fig. 3). Maximum doses of melatonin and noradrenaline were more effec-

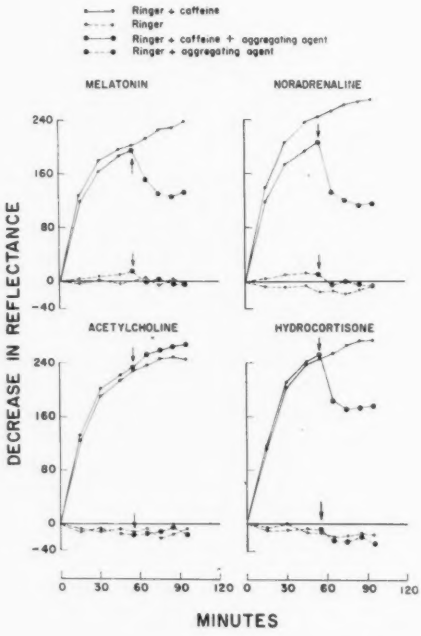


FIG. 3. Skin specimens in Ringer's solution were darkened with caffeine. Aggregating agents were added after 60 minutes. Melatonin, noradrenaline and hydrocortisone all reversed the caffeine darkening, whereas acetylcholine produced a slight increase in darkening.

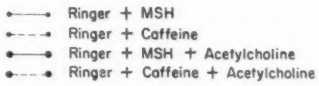
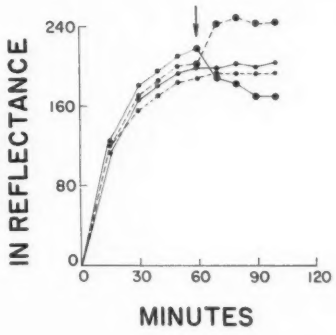


FIG. 4. Of four comparable groups of skin specimens two groups were darkened with caffeine and two with MSH. After 60 minutes acetylcholine was added to one caffeine and one MSH treated group. The caffeine treated group showed a further decrease in reflectance with acetylcholine whereas the MSH treated group lightened.



tive than hydrocortisone (Fig. 2). Acetylcholine in concentrations sufficient to reverse MSH action had no effect on caffeine darkened skin (Fig. 3). To make sure that this inability of acetylcholine to reverse caffeine dispersion did not result from chance use of eight frogs all insensitive to acetylcholine, the same frogs were used to test the action of acetylcholine against MSH and caffeine. Acetylcholine reversed the dispersion caused by MSH but increased the dispersion in specimens from the same frogs darkened by caffeine solution (Fig. 4).

Immersion of skin in hypotonic Ringer's solution produced rapid dark-

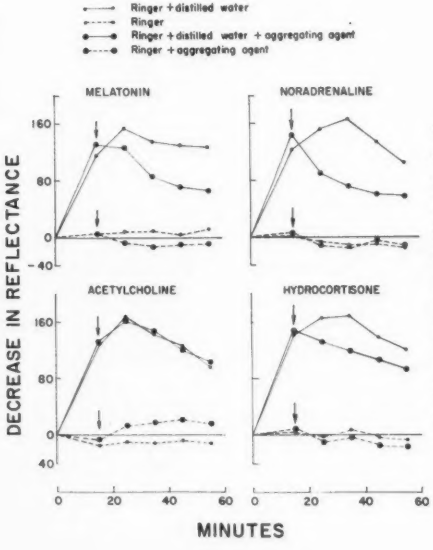


FIG. 5. Skin specimens in Ringer's solution were darkened by diluting the Ringer's solution with distilled water. After 15 minutes, aggregating agents were added. Melatonin, noradrenaline and hydrocortisone reversed the darkening produced by the hypotonic solutions. Acetylcholine had no effect. Control specimens in hypotonic Ringer's reached a maximum darkening in about 30 minutes, and then lightened slightly. Control skins in Ringer's solution showed little change with the addition of aggregating agents except that acetylcholine induced slight darkening.

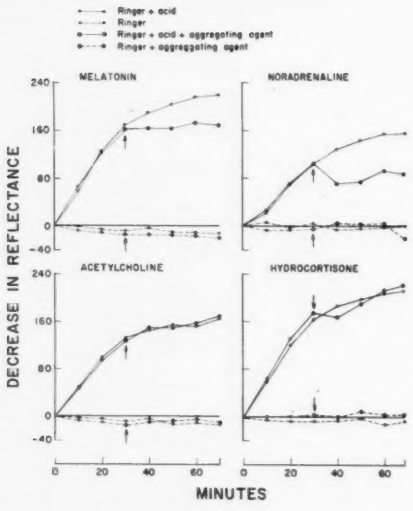
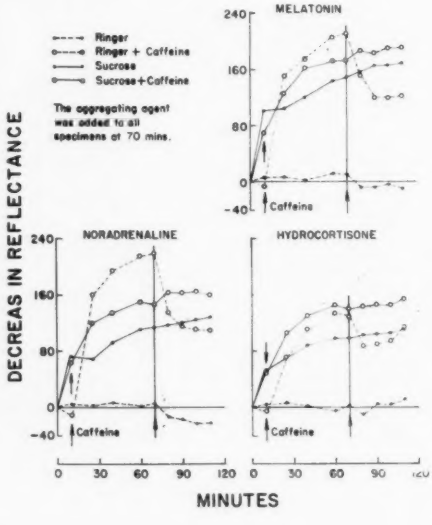


FIG. 6. Skin specimens in Ringer's solution were darkened by acidifying the solution with HCl. Aggregating agents were added at 30 minutes. Melatonin, noradrenaline and hydrocortisone produced a slight reversal of darkening. Acetylcholine had no effect. Control specimens in acidified solution continued to darken slightly. Control specimens in Ringer's solution showed no significant change.

ening. Maximum dispersion occurred in 25 to 30 minutes. Subsequently the granules slowly began to aggregate, and the skin became lighter in color. Melatonin, noradrenaline and hydrocortisone produced an immediate reversal of the dispersion reaction. Acetylcholine did not reverse the dispersion initiated by decreased osmotic activity (Fig. 5). When maximum doses of the lightening agent were used, it was evident that melatonin and noradrenaline were powerful aggregating agents, that hydrocortisone was less active and that its action was not well maintained. In no case was complete reversal of dispersion produced (Fig. 2).

FIG. 7. In each scheme of four groups of skin specimens, two were maintained in Ringer's solution and two in sucrose solution. The latter groups darkened. After 10 minutes caffeine was added to one group in Ringer's solution and one group in sucrose solution. Subsequent darkening due to caffeine was less in the sucrose than in the Ringer's solution. After 70 minutes aggregating agents were added. Melatonin, noradrenaline and hydrocortisone produced no reversal of darkening caused by the sucrose, solutions with or without caffeine. All three aggregating agents reversed the darkening effect of caffeine in Ringer's solution.



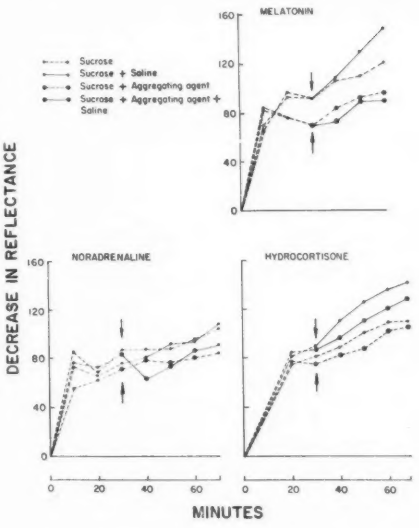


FIG. 8. In each scheme all four groups of skin specimens were placed in sucrose solution. After 30 minutes normal saline was added to one group, aggregating agent to one group, and aggregating agent plus saline to a third group. The control group in sucrose continued to darken slightly as did the group receiving the aggregating agent. The addition of saline alone produced an increase in darkening. When saline and noradrenaline were added together a slight but definite reversal occurred. Melatonin and saline produced a slight lag in the expected darkening. Hydrocortisone and saline had no effect.

Increasing the hydrogen ion concentration of Ringer's solution by adding hydrochloric acid induced dispersion of melanin granules. This dispersion was reversed by noradrenaline but not by melatonin and hydrocortisone when these agents were used in the usual amounts (Fig. 6). However, when maximum quantities of these hormones were used, all reversed the reaction. Only acetylcholine had no effect regardless of concentration (Fig. 2).

When skin was soaked in sucrose solution for ten minutes, variable degrees of darkening occurred. Placing the skin in fresh sucrose solution usually resulted in an immediate slight reversal of dispersion, but then the skin continued to darken. None of the four lightening agents tested reversed the sucrose dispersion effect (Fig. 7) (Fig. 9). Slight reversal of dispersion occurred when 1 ml. of saline was added at the same time as noradrenaline. This effect was not observed with melatonin or hydrocortisone, but melatonin and saline caused a lag in the expected continued dispersion (Fig. 8). It was interesting that in each case the addition of 1 ml. of saline to 20 ml. of sucrose solution increased the darkening beyond that caused by sucrose alone. MSH produced almost no further darkening of skin maintained in sucrose solution (Fig. 9). Caffeine caused further dispersion of pigment granules in skin kept in sucrose solution. However, this added dispersion was less than that produced by the same quantity of caffeine acting on skin kept in Ringer's solution. None of the lightening agents reversed the added darkening brought about by caffeine in sucrose solution Fig. 7).

These findings were in accord with those of Novales (5) that sodium ions are required for MSH action. He also showed that caffeine produced normal dispersion in lithium Ringer's solution whereas MSH had no action. Our experiments confirmed these findings (Figs. 10 and 11). Also melatonin and

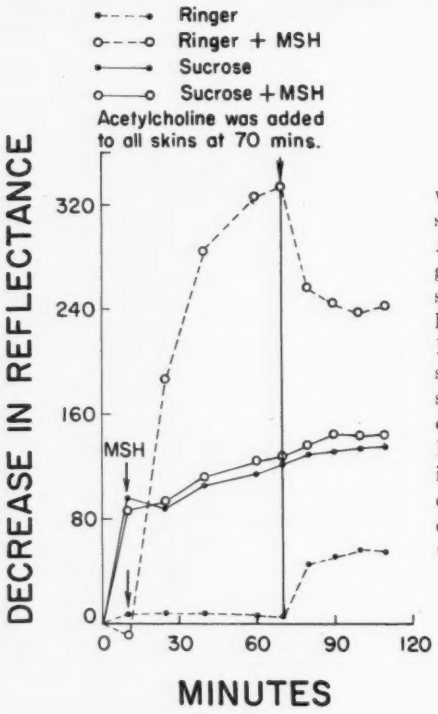


FIG. 9. Two groups of skin specimens were kept in Ringer's solution and two in sucrose solution, the latter darkening. After 10 minutes MSH was added to one group in Ringer's solution and one in sucrose solution. The group in Ringer's solution showed marked darkening, whereas MSH had no effect on the group in sucrose solution. Acetylcholine was added to all specimens after 70 minutes. Reversal of darkening occurred in the MSH and Ringer's solution. The control specimens in Ringer's solution showed a slight darkening reaction. Acetylcholine had no effect on specimens in sucrose solution with or without MSH.

noradrenaline reversed caffeine dispersion in lithium Ringer's solution. Hydrocortisone had little effect—it merely produced a temporary interruption in the otherwise continuing darkening response (Fig. 10).

DISCUSSION

The movement of melanin granules in frog melanocytes is a spectacular phenomenon to observe microscopically. With a variety of treatments granules can be made to move rapidly either from the center of the cell to the periphery or from the periphery to the center. These remarkable reactions can be reversed readily. Granular movement proceeds in an orderly manner. There is no random wandering. Each granule follows its fellow either in a definite outward streaming or in a regulated retreat towards the center of the cell. If the granules are carried in a moving stream of cytoplasm, this movement is in response to definite directional forces.

Seifriz has suggested that all protoplasmic streaming is a response to changing electrical charges within the cell (6). Such changes could be brought about by the movement of charged particles (ions or hydrated ions) between intra- and extracellular compartments. Direct proof of ionic transfer across the cell membrane during dispersion or aggregation of melanin granules must await investigations at a cellular level. However, the

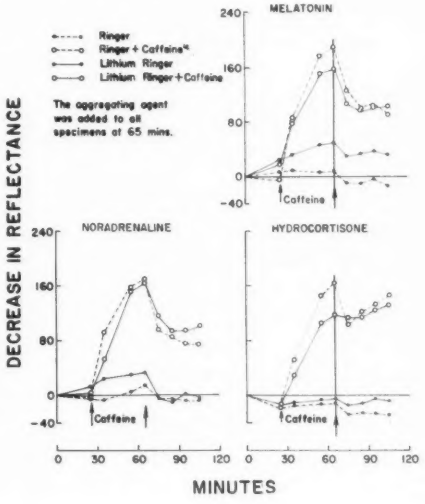


FIG. 10. In each scheme of four groups of skin specimens two groups were in sodium Ringer's solution and two groups in lithium Ringer's solution. There was a slight and variable amount of darkening in the lithium solution. After 20 minutes caffeine was added to one group in lithium and one group in the sodium Ringer's solution. The subsequent darkening was only slightly less in the lithium than in the sodium solution. After 65 minutes the aggregating agents were added to all groups in each scheme. Melatonin and noradrenaline reversed caffeine darkening in both Ringer's solution. Hydrocortisone reversed caffeine darkening in sodium but not in lithium Ringer's solution.

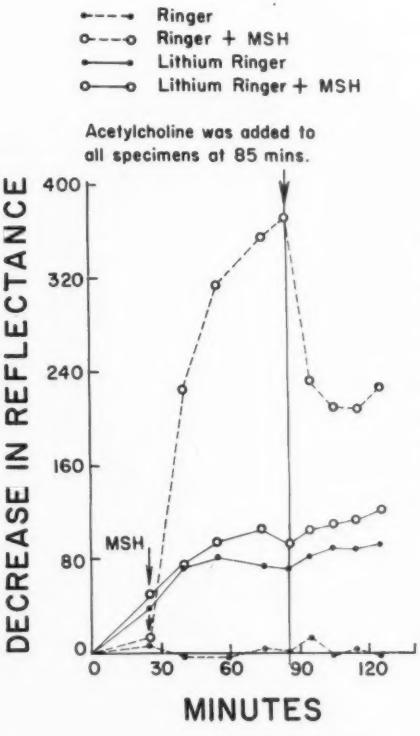


FIG. 11. Two groups of specimens were kept in sodium and two groups in lithium Ringer's solution. The latter two groups showed slight darkening. After 20 minutes MSH was added to one group in lithium and one in sodium Ringer's solution. MSH had no effect in lithium Ringer's solution whereas in sodium Ringer's solution it produced marked darkening. After 85 minutes acetylcholine was added to all specimens. This produced a reversal of darkening in the MSH and sodium Ringer's group. There was no effect in the other three groups.

results of our experiments with pieces of intact skin strongly suggest that ionic transfer is involved. If the composition of Ringer's solution surrounding the skin is changed by adding distilled water, altering the normal ratio of electrolytes (5) or increasing the hydrogen ion concentration, the normal balance between intra- and extracellular fluids is upset and changes are observed in the state of dispersion of melanin granules.

The fact that MSH cannot produce its characteristic effect in the absence of sodium ions indicates that the latter are involved in this specific mechanism of dispersion.

Melanocytes are derived from the neural crest and migrate to their positions in the skin during embryonic development. Noradrenaline and acetylcholine, both active agents in melanin granule aggregation, are chemical transmitters of nerve impulses. It has been shown experimentally that during transmission and conduction of a nerve impulse there is an exchange of potassium and sodium ions accompanying the change in charge across the cell membrane (7). In the absence of adrenal steroids the refractory period of nerve is unduly prolonged. It is during this period that there is a return of potassium ions to the interior of the axon. Melatonin is a 5-methoxyindole derivative present in nerve tissue (10) and is the most potent lightening agent known. All these various points suggest that the specific actions of noradrenaline, acetylcholine, hydrocortisone and possibly melatonin on nerve cells are similar to their actions on melanocytes.

Aggregation and dispersion of melanin granules are probably not produced by enzymic processes, but the relocation of these granules may affect some enzymic activities. There is good evidence that these granules, which are mitochondrial elements, not only contain melanin but also have various enzymic functions (12). Their movement in response to environmental changes is obvious because of their pigment content. The forces which move these granules presumably move other cytoplasmic inclusions that are not so easily identifiable in living unstained preparations. Thus substances known to influence the movement of melanin granules may affect many enzymic activities of the melanocytes and possibly of other cells.

Acknowledgments

We wish to acknowledge the considerable help and statistical work of Dr. Morris Foster and Dr. Colin White which enabled us to devise a satisfactory experimental plan for the simultaneous recording of the changes in four comparable groups of skin specimens.

REFERENCES

1. Schizume, K., A. B. Lerner and T. B. Fitzpatrick: *Endocrinology* **54**: 553. 1954.
2. Foster, M. and M. R. Wright: Unpublished data.
3. Lerner, A. B., J. D. Case, Y. Takashashi, T. H. Lee and W. Mori: *J. Am. Chem. Soc.* **80**: 2587. 1958.
4. Mori, W. and A. B. Lerner: In press.
5. Novales, R. R.: *Physiological Zoology* **32**: 15. 1959.

6. Seifriz, W.: *Nature* **171:** 1136. 1953.
7. Huxley, A. F.: *Ion Transport Across Membranes.* Academic Press, Inc., New York, p. 23. 1954.
8. Hodgkin, A. L.: *Biol. Revs.* **26:** 339. 1951.
9. Van Hof-Van Duin, J.: *Acta Physiologica et Pharmacologica Neerlandica* **7:** 289. 1958.
10. Lerner, A. B., J. D. Case, W. Mori and M. R. Wright: *Nature* **183:** 1821. 1959.
11. Woods, M. W., and J. C. Hunter: *Pigment Cell Biology.* Academic Press, Inc., New York. p. 455. 1959.
12. Hochstein, P.: *Pigment Cell Biology.* Academic Press, Inc., New York, p. 445. 1959.

TIME COURSE OF URINARY EXCRETION OF INORGANIC PHOSPHATE BY RATS AFTER PARATHYROIDECTOMY AND AFTER INJECTION OF PARATHYROID EXTRACT[1]

ERNST H. BEUTNER[2] AND PAUL L. MUNSON
with the Technical Assistance of SYLVIA BATEMAN

Biological Research Laboratories, Harvard School of Dental Medicine and Department of Pharmacology, Harvard Medical School, Boston, Massachusetts

ABSTRACT

Spontaneously voided urine was collected at intervals of 5 to 15 minutes from unanesthetized male rats in metabolic restraining cages during continuous intravenous infusion of physiological salt solution and analyzed for inorganic phosphate (P_i). The rate of excretion of P_i fell within 25 to 52 minutes after parathyroidectomy and continued to decline during the next 3 to 5 hours. At the end of this interval the intravenous injection of parathyroid extract resulted, within 8 minutes, in a rise in urinary P_i. The rate continued to increase to a maximum, 18 to 48 minutes after the injection, which was within the range of the preoperative values. The rate fell gradually toward the pre-injection level during the next one to one and a half hours. The work led to the following conclusions: the life of parathyroid hormone in the circulation is short; in the normal intact rat there is a continuous secretion of the hormone; the hormone acts directly on the renal tubule to influence the excretion of P_i. The bearing of the data on concepts of the mode of action of parathyroid hormone is discussed.

RECENT research, reviewed by Greep and Kenny (1), has necessitated a re-evaluation of the hypothesis of Albright and co-workers (2, 3) that the parathyroid hormone exerts its effect on calcium metabolism primarily through its action on phosphorus metabolism, both renal and extrarenal. It has now been clearly demonstrated that parathyroid extract can raise the blood calcium in the absence of the kidneys (4, 5, 6), that parathyroid implants result in a marked localized resorption of bone (7, 8), and that changes in serum calcium after parathyroidectomy or administration of parathyroid extract occur much earlier than any opposite change in the serum inorganic phosphate (9). These results in experimental animals, as well as observations in the clinic (10, 11) that are also at variance with

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[2] Present address: Department of Bacteriology and Immunology, School of Medicine, University of Buffalo, Buffalo 14, New York.

Albright's hypothesis, have made it conceivable that the apparent effect of parathyroid hormone on the rate of urinary excretion of phosphate is an artifact (5) or of questionable physiological significance. The original discoveries of Greenwald and Gross (12, 13) are, nevertheless, still valid. The parathyroid hormone has a definite, although incompletely analyzed, effect on the renal excretion of inorganic phosphate (14, 15, 16), and it remains to place this effect and the direct effect on bone in proper perspective for a clearer understanding of the role of the parathyroids in the regulation of the metabolism of calcium and phosphorus.

Observation of the sequence of metabolic changes that occur after the performance of an experimental procedure constitutes a powerful method for the investigation of biological mechanisms. Thus, in a previous study (9), the order of appearance of changes in serum calcium and inorganic phosphate after parathyroidectomy and administration of parathyroid extract helped to clarify concepts about the mode of action of parathyroid hormone. In the present investigation, the time course of the changes in rate of urinary excretion of inorganic phosphate by the rat following the same two treatments was determined. From the results, it was possible to make certain inferences concerning the rapidity of loss of parathyroid hormone from the circulation, the continuity of secretion of hormone by the glands, and the renal tubule as a primary site of action of the hormone.

MATERIALS AND METHODS

The experimental animals were male albino rats of an inbred strain obtained commercially (Holtzman Co., Madison, Wis.). After receipt from the supplier and until the beginning of the experiment, the rats were housed in suspended wire mesh cages in an air-conditioned animal room maintained at 78° ± 2° C and were exposed to 12-hour light-dark intervals. The rats were fed a commercial diet (Purina laboratory chow) and tap water *ad lib.* Before the experiment the rats were placed in the plastic restraining cages of Kellogg *et al.* (17) for several hours on successive days in order to accustom them to the environment of the experiment. The design of the restraining cage permits separate collection of urine and feces and continuous intravenous infusion through a tail vein without anesthesia (except during the initial cannulation).

The test rat was placed in the restraining cage the evening before the day of experiment and immediately following cannulation of a tail vein. Continuous intravenous infusion of physiological salt solution at a rate of approximately 0.2–0.4 ml./min. was then begun and continued during the night and throughout the experiment the following day. Micturition occurred spontaneously every 5 to 15 minutes. Each urine sample was collected separately and analyzed for inorganic phosphate by the method of Fiske and SubbaRow (18). The time intervals between and the volumes of successive micturitions were recorded, and the rate of phosphate excretion was calculated as μg. of inorganic phosphorus (P_i) per minute. The rat was given no food to eat and no water to drink from the beginning of the intravenous infusion.

For parathyroidectomy the rat was taken from the cage without removal of the intravenous cannula and anesthetized with ether. The glands were destroyed by cautery with a hot wire (details of the operation on request), the incision was closed, and the rat was returned to the cage. Only about 5 to 10 minutes was required for this procedure. In 4 rats a sham operation, which involved ether anesthesia, an incision, exposure of the

thyroid, and cautery of adjacent muscles, was performed 103–147 minutes before parathyroidectomy.

A crude hot or cold acid parathyroid extract prepared in the laboratory from frozen bovine parathyroid tissue[3] was injected into the intravenous cannula. The extracts were biologically standardized by the method of Munson *et al.* (9, 19). In 3 rats a control injection of physiological salt solution acidified to pH 2 was also made.

RESULTS

Effect of parathyroidectomy on urinary inorganic phosphate

Five rats were parathyroidectomized after 8 to 16 hours of continuous intravenous infusion with physiological salt solution; the effect on the rate of excretion of P_i is summarized in Table 1.

The control rate of excretion of P_i was measured for 80–160 minutes immediately preceding ether anesthesia and a sham parathyroidectomy (in

TABLE 1. EFFECT OF PARATHYROIDECTOMY ON RATE OF URINARY EXCRETION OF INORGANIC PHOSPHORUS BY RATS

Rat No.	Age	Body weight	Preop. P_i† mean ± S.D.	Time after PTX for P_i to fall below preop. mean − S.D.	Time after PTX for P_i to fall below 15 μg./min.
	days	gm.	μg./min.	min.	min.
1	202	425	60 ± 4.0	25	118
2	63	246	36 ± 2.8	38	84
3	83	312	28 ± 2.5	49	95
4	56	220	24 ± 4.6	52	58
5	91	320	33 ± 6.6	29	47
6*	52	167			

Abbreviations: preop., preoperative; P_i, inorganic phosphorus; S.D., standard deviation; PTX, parathyroidectomy.

* Data from rat no. 6 are in Table 2.

† Period of 80–160 min. immediately preceding surgery.

4 of the 5 rats). The mean control rate was computed by dividing the μg. of P_i excreted during the entire period by the total duration of the period in minutes. The mean of the values for the individual urine collections, ignoring differences in time intervals between collections, was less than 2.4% different from the true mean rate in all rats. The estimate of the "standard deviation" was unavoidably computed from the unweighted individual values. This departure from sound statistical practice does not appear to have affected the conclusions in any important way.

Following the sham operation, there was an immediate transitory fall in the rate of urine flow and excretion of P_i, followed by an "overshoot" in the opposite direction, with a gradual return to the control level. This consistent observation is illustrated in Figure 1 with data from rat no. 1.

A similar transitory, biphasic, artifactual response was observed immediately after parathyroidectomy, which was performed 103–147 minutes after the sham operation. However, in 25 to 52 minutes after parathy-

[3] Kindly donated by Eli Lilly and Co., through the courtesy of Dr. K. K. Chen.

roidectomy, the rate of P_i excretion had fallen one standard deviation or more below the control mean. In a relatively short additional time, of 6–93 minutes, the urinary P_i of all the rats had fallen below 15 μg./minute. Because of the considerable variation in age and body weight of the experimental animals, the data, although rather consistent, were not averaged.

Effect of intravenous parathyroid extract on urinary inorganic phosphate

Observations of the phosphaturic response to intravenous injection of PTE are summarized in Table 2. The injection was made 3 to 5 hours after

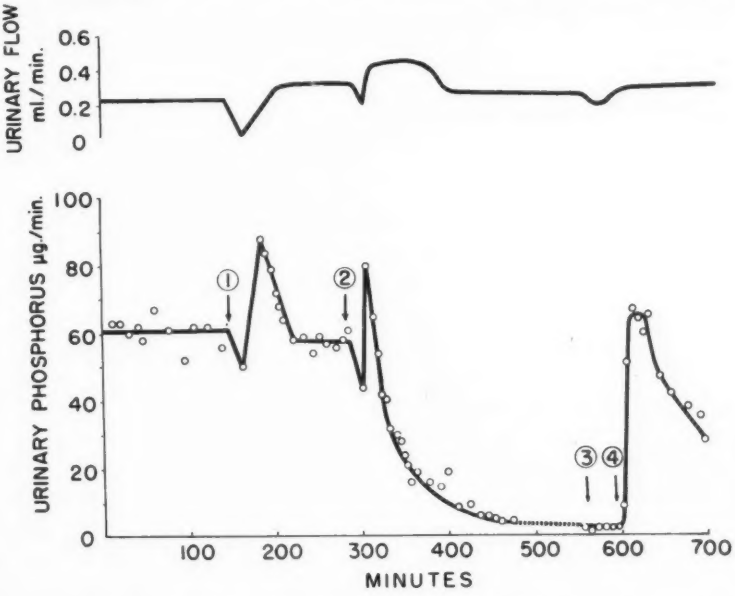

FIG. 1. Time course of urinary excretion of inorganic phosphate by an unanesthetized rat after parathyroidectomy and after injection of parathyroid extract, in comparison with appropriate control treatments. At arrow 1, the rat was removed from its restraining cage, anesthetized with ether, and subjected to a sham operation. At arrow 2, the rat was again anesthetized with ether and parathyroidectomized. At arrow 3 acidified physiological salt solution was injected intravenously. At arrow 4 parathyroid extract was injected intravenously.

parathyroidectomy except for rat no. 6 (15 hours). Therefore, the rate of excretion of P_i had already fallen to a low level at the time of the injection, although in most cases a downward trend was still evident.

The parathyroid extract was acidic (pH 2–3) in all cases but no. 3, in which it was neutralized before injection. In three experiments, a control intravenous injection of an equal volume (0.10 or 0.22 ml.) of acidified physiological saline was made 20–120 minutes before the injection of PTE. The control injection had no apparent effect on the excretion of P_i.

TABLE 2. EFFECT OF INTRAVENOUS INJECTION OF PARATHYROID EXTRACT ON RATE OF URINARY EXCRETION OF INORGANIC PHOSPHORUS BY ACUTELY PARATHYROIDECTOMIZED RATS

Rat No.*	Dose of PTE	P_i before injection† mean ±S.D.	Time after PTE for P_i to rise above pre-inj. mean+S.D.	Maximum P_i after PTE	Time after PTE to maximum P_i
	U./rat	μg./min.	min.	μg./min.	min.
1	20	5.8±1.8	7	67	23
2	10	12.1±1.8	7	33	18
3	20	9.2±1.6	5	26	44
4	10	10.7±0.8	8	22	48
6	20	16.3±4.2	0.3‡	66	46

Abbreviations: PTE, parathyroid extract; U., units; pre-inj., pre-injection; also see Table 1.

* See Table 1 for age and body weight.

† Period of 30–60 min. immediately preceding injection.

‡ Spontaneous micturition occurred 20 seconds after the injection of PTE. The observed increase in P_i may have been a chance occurrence.

In contrast, within 1 to 8 minutes after the injection of 10 or 20 units of parathyroid extract, the rate of excretion of P_i rose at least one standard deviation above the pre-injection mean, as shown in Table 2. In most cases, the time interval between the injection and the rise in urinary P_i was undoubtedly even shorter than indicated in the table, if it may be assumed that the rise began to occur before it was detected at the end of the urine collection period.

The rate of excretion of P_i continued to rise rather quickly, in 22 to 67 minutes, to a maximum that was in the range of the values before parathyroidectomy in the experiments (rats no. 1–4) in which these values had been determined. The period of maximum excretion of P_i was rather brief and the P_i promptly began to fall again, although more gradually than it had risen. The present experiments were terminated one and a half to two hours after the injection, and at this time the urinary P_i had not yet fallen to the pre-injection level.

Figure 1, cited above for data on the effect of a sham operation, shows an illustrative experiment (rat no. 1) in which the rate of excretion of P_i was measured almost continuously for approximately 12 hours, before and after sham operation, parathyroidectomy, control injection, and injection of parathyroid extract.

DISCUSSION

Our previous conclusion from observation of the rapid fall in serum calcium after parathyroidectomy (9, 20) that the parathyroid hormone in the circulation is short-lived (21) is supported by the present data on urinary P_i. Both sets of observations also suggest that in the normal intact rat parathyroid hormone is secreted more or less continuously.

The response of the kidney within a few minutes after intravenous injection of parathyroid extract suggests that parathyroid hormone acts

directly on the renal tubule. Although clearance techniques were not employed, the magnitude of the changes observed was so great that it seems inconceivable that they could have been due in more than small part to hemodynamic factors. A similar conclusion was reached by Gershberg *et al.* (22), who studied the response to intravenous parathyroid extract in 42 human patients. Glomerular filtration rate, renal plasma flow, and plasma and urinary P_i were determined simultaneously, and it was concluded that in all the patients except two with hyperparathyroidism the per cent of filtered P_i reabsorbed fell within 15 minutes after the injection. The establishment of a direct effect of parathyroid hormone on the renal tubule would not rule out the possibility, recently suggested by Foulks and Perry (15, 23, 24), that an important function of parathyroid hormone is "the maintenance of a normal distribution of inorganic phosphate in body fluids" through extrarenal actions on cellular phosphate metabolism.

Previously (9), we have emphasized the fact that, contrary to the hypothesis of Albright and Ellsworth (2, 3), after parathyroidectomy and after injection of parathyroid extract changes in serum calcium occur more rapidly than opposite changes in serum P_i. Our present report demonstrates that changes in the urinary P_i occur much more rapidly than changes in either the calcium or P_i of the serum. The fall and rise in urinary P_i observed in response, respectively, to parathyroidectomy and parathyroid extract, although large in comparison with the appropriate control periods, were small in relation to the total available P_i. Only after a considerable period of time would these changes exert sufficient effect, especially in the face of various compensating mechanisms, to be reflected in detectable changes in serum P_i. The observation of rapid changes in urinary P_i is therefore not inconsistent with previous data (9) that demonstrated a considerable delay in the response of the serum P_i to parathyroidectomy and to parathyroid extract.

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REFERENCES

1. Greep, R. O. and A. D. Kenny in G. Pincus and K. V. Thimann (eds.) The Hormones, v. 3. Academic Press, New York, 1955, pp. 153–174.
2. Albright, F. and R. Ellsworth: *J. Clin. Invest.* **7:** 183. 1929.
3. Albright, F. and E. C. Reifenstein, Jr.: The Parathyroid Glands and Metabolic Bone Disease. Williams and Wilkins Co., Baltimore, 1948.
4. Grollman, A.: *Endocrinology* **55:** 166. 1954.
5. Stewart, G. S. and H. F. Bowen: *Endocrinology* **48:** 568. 1951.

6. TALMAGE, R. V., F. W. KRAINTZ, R. C. FROST AND L. KRAINTZ: *Endocrinology* **52**: 318. 1953.
7. BARNICOT, N. A.: *J. Anat.* **82**: 233. 1948.
8. CHANG, H.: *Anat. Record* **111**: 23. 1951.
9. MUNSON, P. L.: *Ann. N. Y. Acad. Sci.* **60**: 776. 1955.
10. KRANE, S. M.: *J. Clin. Endocrinol.* **17**: 386. 1957.
11. CANARY, J. J., H. A. BLOOMER, G. C. CARREON AND L. H. KYLE: The Endocrine Society, Program of the Forty-first Meeting, p. 28. 1959.
12. GREENWALD, I.: *Am. J. Physiol.* **28**: 103. 1911.
13. GREENWALD, I. AND J. GROSS: *J. Biol. Chem.* **66**: 217. 1925.
14. HIATT, H. H. AND D. D. THOMPSON: *J. Clin. Invest.* **36**: 557. 1957.
15. FOULKS, J. G. AND F. A. PERRY: *Am. J. Physiol.* **196**: 567. 1959.
16. NICHOLSON, T. F.: *Canad. J. Biochem. & Physiol.* **37**: 113. 1959.
17. KELLOGG, R. H., W. R. BURACK AND K. J. ISSELBACHER: *Am. J. Physiol.* **177**: 27. 1954.
18. FISKE, C. H. AND SUBBA ROW, Y.: *J. Biol. Chem.* **66**: 375. 1925.
19. MUNSON, P. L., A. D. KENNY AND O. A. ISERI: *Federation Proc.* **12**: 249. 1953.
20. MUNSON, P. L., O. A. ISERI, L. W. PARKER AND R. O. GREEP: *J. Dental Research* **31**: 463. 1952.
21. MUNSON, P. L., A. D. KENNY, O. A. ISERI AND R. O. GREEP: *J. Dental Research* **32**: 670. 1953.
22. GERSHBERG, H., D. R. SHIELDS AND S. S. KOVE: *J. Clin. Endocrinol.* **19**: 681. 1959.
23. FOULKS, J. G. AND F. A. PERRY: *Am. J. Physiol.* **196**: 554. 1959.
24. FOULKS, J. G. AND F. A. PERRY: *Am. J. Physiol.* **196**: 562. 1959.

BIOSYNTHESIS OF STEROIDS IN STALLION TESTIS TISSUE[1,2]

KENNETH SAVARD[3] AND JOSEPH W. GOLDZIEHER

Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts, and the Department of Endocrinology, Southwest Foundation for Research and Education, San Antonio, Texas

ABSTRACT

The testis of a gonadotropin-stimulated stallion was perfused with horse blood containing sodium acetate-1-C^{14} for 24 hours. The neutral lipide fraction (with the aid of carrier steroids) on fractionation and purification by conventional methods including chromatography on paper, crystallization and formation of derivatives, yielded the following radiochemically pure substances: 4-androstene-3,17-dione, testosterone, progesterone and 17-hydroxyprogesterone. 11β-Hydroxy-4-androstene-3,17-dione, added as carrier, was isolated devoid of radioactivity, signifying its absence in normal testis tissue. The high level of isotope incorporated into the above steroids is interpreted as reflecting a relatively great steroidogenic capacity of stallion testis tissue.

THE recent elucidation of the biosynthetic pathway leading to the androgenic steroid hormones in rat, bovine and human testis tissue (1–4), has revealed the role of progesterone as the biochemical precursor of testosterone and 4-androstene-3,17-dione, both of which are formed in and secreted by testis tissue (5–8). The pathway postulated by Slaunwhite and Samuels (1) (progesterone→17-hydroxyprogesterone→4-androstene-3,17-dione⇄testosterone), and demonstrated by means of isotopically-labelled progesterone and 17-hydroxyprogesterone (1–4, 9–10) added as substrate, requires that both progesterone and 17-hydroxyprogesterone be formed endogenously in normal testis tissue. Both compounds have been shown to be synthesized (from acetate-1-C^{14}) in testicular tumor tissues (9, 11) and have been isolated from normal ovarian (12) and arrhenoblastoma tissues (13).

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[3] Investigator, Howard Hughes Medical Institute; present address: Endocrine Laboratory, Department of Medicine, University of Miami School of Medicine, Miami, Fla.

In the present study we wish to report the endogenous formation of both radioactive progesterone and 17-hydroxyprogesterone, as well as radioactive 4-androstene-3,17-dione and testosterone from acetate-1-C^{14} by an intact gonadotropin-stimulated stallion testis. By means of the carrier-dilution technique the *relative* amounts of the four steroids formed by the perfused testis have also been determined from the accumulation of isotope in each steroid.

EXPERIMENTAL AND RESULTS

The detailed description of the pre-treatment (130,000 I.U. of chorionic gonadotropin, A.P.L., Ayerst Laboratories, New York) of the stallion, the orchiectomy and perfusion of the testis, have been reported (14). Briefly the procedure consisted of the administration of gonadotropin for 6 days. On the seventh day the testis was removed under pentothal anesthesia, and the artery cannulated. The intact gland was perfused continuously for 24 hours with a sterile solution of defibrinated horse blood and White's solution (equal parts) to which were added 2.0 mc. of sodium acetate-1-C^{14}. At the end of the perfusion, the testis tissue and blood (as plasma and cells) were treated separately as described earlier (14). The neutral lipide fractions were combined to provide a total extract weighing 1.027 gm. and containing 125×10^6 c.p.m. as determined in a flow-gas windowless counter operating at approximately 45 per cent efficiency. At this point crystalline steroids were added as carriers in order to facilitate the isolation of radioactive substances present in the lipide extract; all steroids were homogeneous as determined by paper chromatography, and the amounts added are listed below.

The mixture of carrier steroids and crude neutral lipides was subjected to an 8-transfer counter-current distribution between ligroin (b.p. 60–70°) and 90 per cent methanol-water in which the lower phase (aqueous methanol) was transferred. The resulting distribution of weight and radioactivity among the 9 tubes are shown in Table 1. Only tubes 7 and 8 contained significant amounts of the carrier steroids as determined by absorption at 240 mμ; the contents of these tubes were combined, dissolved in toluene and extracted several times with 1N sodium hydroxide in order to remove traces of radioactive acidic and phenolic substances. The neutral fraction was treated with Girard's reagent T (15) to provide a ketonic neutral and a non-ketonic neutral fraction; the separation was carried out once more on the non-ketonic fraction. The distribution of radioactivity and weight among these fractions is given in Table 1.

The purified neutral ketonic extract was subjected to chromatography on filter paper in the system ligroin/propylene glycol (16) according to the following scheme: the extract was chromatographed on six 16-cm. wide sheets for 98 hours, to provide two steroid zones (located by examination under ultraviolet light), the slower of which was 11β-hydroxy-4-andro-

stene-3,17-dione and the faster containing both 17-hydroxyprogesterone and testosterone in admixture (16). The steroid zones were eluted separately and rechromatographed in the same solvent system in order to remove effectively traces of other steroids which were present in consequence of the heavy loading of the initial chromatograms. The portion of the extracts which had moved off the paper chromatograms (run-off) was chromatographed in the same system for 17 hours to provide two steroid zones, 4-androstene-3,17-dione and progesterone; each steroid zone was eluted and separately chromatographed once more.

The double zone containing 17-hydroxyprogesterone and testosterone was freed from propylene glycol by dissolving in methylene chloride and shaking several times with water. The solvent was removed and the residue

TABLE 1. DISTRIBUTION OF WEIGHT AND RADIOACTIVITY IN FRACTIONATION OF NEUTRAL LIPIDE EXTRACT

	mg.	c.p.m.
Total lipide extract (+added carriers)	1,181	125 $\times 10^6$
8-Transfer c.c.d.		
tube #0	579	48.15×10^6
1	173	33.15×10^6
2	53	18.20×10^6
3	36	6.15×10^6
4	22	2.73×10^6
5	17	1.17×10^6
6	36	2.09×10^6
7	61	2.89×10^6
8	160	9.47×10^6
Tubes 7+8 Non-ketonic fraction	32.5	2.04×10^6
Tubes 7+8 Ketonic fraction	152.9	8.16×10^6

dried (16.4 mg.); treatment with acetic anhydride and pyridine (17) afforded a residue which when chromatographed in ligroin-propylene glycol (8 hours) separated into two steroid zones, the slower of which was 17-hydroxyprogesterone and the faster was testosterone acetate.

Each steroid so obtained was free from other steroids, but contained considerable amounts of lipide and non-specific radioactive materials. Repetition of chromatography on paper eventually provided semi-crystalline products which were further purified on small columns of silica gel (Davison Chemical Company, Baltimore, Maryland) by elution with benzene containing increasing amounts of ethyl acetate. The final products were measured by weight or by absorption at 240 mμ in methanol; recovery of the added carrier steroids at this stage varied between 2.0 and 6.0 mg. Since the steps leading to radiochemical purity of these products differed somewhat in each case, each will be described separately.

Progesterone. Two milligrams were obtained with a total radioactivity content of approximately 64,000 c.p.m. This was diluted with 18 mg. of

additional crystalline progesterone carrier and recrystallized several times from methanol. A final crop of crystals weighed 5.0 mg. (m.p. 122–123° uncorr.) and had a specific activity[4] of 2,025; the non-crystalline residue in the mother liquors counted 1,920 per mg. Formation of the oxime did not change the specific activity.

17-Hydroxyprogesterone. Two milligrams of crystalline product were obtained from the chromatograms following the separation from testosterone acetate. Addition of carrier (15 mg.) and chromatography in methyl cyclohexane:toluene (1:1 by volume)-propylene glycol (8 hours) gave a single zone which after several recrystallizations from methanol yielded 7.0 mg. of crystalline 17-hydroxyprogesterone (m.p. 216–217°, uncorr.), specific activity 240 and a mother liquor residue whose specific activity was 257. Formation of the oxime, chromatography on paper in toluene-propylene glycol (70 hours) and crystallization did not change the specific activity.

4-Androstene-3,17-dione. The product was obtained as an oil and measured 2.2 mg. by absorption at 240 mμ in methanol and contained a total of 169,500 c.p.m. Fifteen milligrams of carrier steroid were added and the mixture chromatographed in ligroin-propylene glycol to yield a single zone, which on elution and silica gel chromatography yielded a crystalline residue. This was crystallized several times from hexane before a mother liquor residue and the derived crystals both had the same specific activity (within experimental error) of 7,300 and 7,000, respectively. The crystals were used in the formation of the oxime and the product showed no change in specific activity.

Testosterone. A total of 3.5 mg. of testosterone acetate, containing 324,000 c.p.m., was obtained following chromatography. This was recrystallized from hexane to yield 1.3 mg. (m.p. 135–137°, uncorr.) with a specific activity of 34,000 (calculated as testosterone). To 1.07 mg. of this were added 15 mg. of crystalline testosterone acetate and the mixture recrystallized several times from hexane to yield crystals (9.4 mg.) and mother liquor residue, both counting 2,280 per mg. Saponification of the crystals in methanolic potassium bicarbonate gave testosterone with a specific activity of 2340 after crystallization from methanol.

11β-Hydroxy-4-androstene-3,17-dione. As obtained from the chromatograms, this compound weighed 2.0 mg. and had a specific activity of 4,350. This was diluted with 15 mg. crystalline 11β-hydroxy-4-androstene-3,17-dione and recrystallized several times from acetone-hexane to yield a final crop of crystals (m.p. 198–199° corr., specific activity 61) and a mother

[4] Specific Activity (counts per minute per mg.) was determined in duplicate on 200–800 μg. samples (microbalance) of steroid plated on tared aluminum dishes; a minimum of 4,000 counts was accumulated for each sample. Where derivatives of steroids were measured the given specific activities were corrected for differences in molecular weight and calculated for the free steroid.

liquor residue of specific activity, 370. Oxidation in chromic acid-acetic acid gave adrenosterone which was chromatographed on paper in ligroin-propylene glycol (41 hours), and recrystallized from methanol. The crystals (m.p. 218–219° corr.) had a specific activity of 19; the mother liquor residue counted 52 per mg. Formation of the oxime and crystallization gave a product with no significant radioactivity content. The radioactivity associated with the carrier 11β-hydroxy-4-androstene-3,17-dione was therefore considered to be due to contamination.

The final specific activities of each of the above compounds at the stage of radiochemical purity (except 11β-hydroxy-4-androstene-3,17-dione), represent values on material diluted a second time with carrier steroid.

TABLE 2

Carrier steroid added	mg.	Final derived specific activity* c./m./mg.	Total c.p.m.	Percent of acetate-1-C^{14} added as substrate**
progesterone	25.0	20,250	0.506×10^6	0.022
17-hydroxyprogesterone	26.6	2,040	0.054×10^6	0.002
4-androstene-3,17-dione	25.4	59,500	1.500×10^6	0.068
testosterone	25.0	34,400	0.860×10^6	0.039
11β-hydroxy-4-androstene-3,17-dione	26.7	nil	nil	—

* Values in this column are based upon actual values obtained following further dilution with carrier steroids; for details see experimental section.

** Based upon a counting efficiency of 45 per cent.

From the final specific activity value, the quantity of diluent steroid and the quantity which was diluted, the specific activity of each original carrier added at the initial stage of the study was calculated. This derived specific activity multiplied by the initial quantity (in mg.) of carrier, provides the total accumulation of isotope in each steroid. Both derived specific activities and total radioactivities are given in Table 2, along with the weights of carrier steroids added at the outset of the study.

DISCUSSION

The incorporation of acetate-1-C^{14} by surviving endocrine tissue preparations into various steroid products is a well accepted qualitative means of assessing hormonal anabolism. By entering into the synthetic process at an early point, the radioactive precursor is carried through the steps of steroid biosynthesis to accumulate in the normal end-products. The carrier-dilution technique provides a means of studying the total radioactivity accumulated in certain specific steroid products, and if carried out quantitatively, affords a measure of the *relative* amounts of the steroids formed in the course of the experiment. The present study therefore has afforded such an assessment of the elaboration of five steroid substances by a stallion testis, in which the endocrine function is presumed to have been substantially enhanced by the gonadotropin administered to the stallion prior to the removal and perfusion of the gland.

The results reveal a significant accumulation of isotope in four of the five steroids studied and provide evidence for their biosynthesis. Thus, as the values in Table 2 show, radioactive 4-androstene-3,17-dione, testosterone, progesterone and 17-hydroxyprogesterone in that order of quantity, have been synthesized from acetate-1-C^{14} by the stallion testis. The two major steroids produced by the gland, testosterone and 4-androstene-3,17-dione, have previously been detected in the effluent of dog (7, 8) and human testes (6); rat testis, however, does not appear to form testosterone endogenously (from radioacetate) (5). The relative amounts of radioactivity in each of the two androgens formed in this study indicate that the stallion testis produces (at least on perfusion) twice as much 4-androstene-3,17-dione as it does testosterone. The reverse, however, is found in canine (8) and bovine testis (Savard and Gassner, unpublished data), suggesting the possibility of a species difference in the ratio of these two companion androgens. It is interesting that the estrogens elaborated and released by this perfused stallion testis and reported separately (14), showed estrone and estradiol-17β to be in approximately the same ratio as the two androgens synthesized simultaneously and reported here.

Radioactivity in the progesterone and 17-hydroxyprogesterone isolated in this study serves to demonstrate for the first time the endogenous synthesis of these two compounds by normal (non-neoplastic) testis tissue. This is the first reported demonstration of the endogenous biosynthesis of 17-hydroxyprogesterone by the male gonad; it has however been isolated from normal ovary (12). Both compounds are synthesized from radioacetate by human testis tumors (9, 11). This evidence of progesterone and 17-hydroxyprogesterone biosynthesis in testis tissue serves to establish firmly the role of these two C_{21} steroids as the biochemical precursors of testicular androgens as first proposed by Slaunwhite and Samuels (1). Together with the results of studies on other gonadal tissues (18–20) and on the adrenal gland (21), this evidence supports the concept of a fundamental, common biosynthetic pathway for the formation of the steroid hormone skeleton, a pathway which is identical in all steroidogenic tissues, with the possible exception (for want of evidence at the present time) of the placenta. The sequence of chemical alterations, from the precursors of progesterone to those end products characteristic of the particular tissue, appears to be qualitatively the same in the gonads and the cortex of the adrenal. This common property of these three tissues may well reflect their related embryological origins. Moreover, this similarity may prove to be even greater than suspected in the light of the finding of 21-hydroxylase in normal rat testis tissue recently reported (22).

The absence of radioactivity in the carrier 11β-hydroxy-4-androstene-3,17-dione isolated in this study implies that this compound, normally produced by the adrenal cortex (23, 24) and recently shown to be elabo-

rated by a virilizing interstitial-cell tumor of the testis (6, 9), is not an important product of the gonadotropin-stimulated testis.

The synthesis of progesterone and 17-hydroxyprogesterone by testis tissue implies an additional source of the precursors of human urinary pregnanediol and pregnanetriol. The former has hitherto been considered to reflect progesterone production by the adrenal and ovary, while the latter has been associated with the abnormal adrenocortical function of congenital adrenal hyperplasia (25). The occurrence of both pregnanediol and pregnanetriol in the urine of the male may now be ascribed in part to the secretion of both progesterone and 17-hydroxyprogesterone by the testis. Although significant, the synthesis of the latter compound relative to that of the two androgens is, however, slight.

In view of the preoperative gonadotropin stimulation, some reservations may be held as to the normalcy of the gland employed. This aspect may however be less important than the changes wrought by the perfusion conditions, which attempt to approximate the natural state of the gland. It is noteworthy in this regard, that in recent studies (Savard and Goldzieher, unpublished data) a stallion testis, prepared and perfused under similar conditions, was capable of the synthesis *de novo* of 6–7 mg. of total androgen (as measured by bioassay). Furthermore, a most impressive incorporation of isotope from acetate-1-C^{14} occurred in the present experiment; the accumulation of 0.13 per cent of the radioactive precursor (Table 2) in the four steroids studied has been observed only in the case of an interstitial-cell tumor of the testis (9) and therefore must reflect the great overall steroidogenic capacity of the stallion testis. The high estrogen output (14), reflected in the urinary estrogen titers of the stallion (26, 27), appears not to be unusual, but rather to indicate a large total steroidogenic ability of the stallion testis.

It should be noted that considerable non-steroid radioactivity found its way through the various steps of the separation procedure (Table 1). This tenacious contamination, if not rigorously eliminated, oftentimes may distort quantitative and qualitative results in this type of study. The isolation of 11β-hydroxy-4-androstene-3,17-dione, devoid of radioactivity, lends increased confidence to the reliability of the methods employed (9).

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REFERENCES

1. Slaunwhite, W. R., Jr. and L. T. Samuels: *J. Biol. Chem.* **220**: 341. 1956.
2. Savard, K., R. I. Dorfman, B. Baggett and L. L. Engel: *J. Clin. Endocrinol. and Metab.* **16**: 1629. 1956.
3. Lynn, W. S., Jr. and R. H. Brown: *Biochim. et biophys. acta* **21**: 403. 1956.

4. Viscelli, T. A., M. E. Lombardo and P. B. Hudson: *Fed. Proc.* **16:** 265. 1957.
5. Brady, R. O.: *J. Biol. Chem.* **193:** 145. 1951.
6. Savard, K., R. I. Dorfman and E. Poutasse: *J. Clin. Endocrinol. and Metab.* **12:** 935. 1952.
7. West, C. D., V. P. Hollander, T. H. Kritchevsky and K. Dobriner: *J. Clin. Endocrinol. and Metab.* **12:** 915. 1952.
8. Brink-Johnsen, T. and K. Eik-Nes: *Endocrinology* **61:** 676. 1957.
9. Savard, K., R. I. Dorfman, B. Baggett, L. L. Fielding, L. L. Engel, H. T. McPherson, L. M. Lister, D. S. Johnson, E. C. Hamblen and F. L. Engel: *J. Clin. Invest.* **39:** 534. 1960.
10. Lynn, W. S., Jr. and R. H. Brown: *J. Biol. Chem.* **232:** 1015. 1958.
11. Wotiz, H. H., J. W. Davis and H. M. Lemon: *J. Biol. Chem.* **216:** 677. 1955.
12. Zander, J.: *J. Biol. Chem.* **232:** 117. 1958.
13. Anliker, R., O. Rohr and L. Ruzicka: *Annalen der Chemie* **602:** 109. 1957.
14. Nyman, M. A., J. Geiger and J. W. Goldzieher: *J. Biol. Chem.* **234:** 16. 1959.
15. Girard, A. and G. Sandelescu: *Helv. chim. Acta* **19:** 1095. 1936.
16. Savard, K.: *J. Biol. Chem.* **202:** 457. 1953.
17. Reichstein, T. and J. von Euw: *J. Helv. chim. Acta* **21:** 1181. 1938.
18. Solomon, S. S., R. V. Wiele and S. Lieberman: *J. Am. Chem. Soc.* **78:** 5453. 1956.
19. Savard, K., R. I. Dorfman, J. L. Gabrilove and L. J. Soffer: 39th Meeting of the Endocrine Society, New York, N. Y. May 30–June 1, 1957.
20. Solomon, S. S., J. T. Lanman, J. Lind and S. Lieberman: *J. Biol. Chem.* **233:** 1084. 1958.
21. Hechter, O., A. Zaffaroni, R. P. Jacobsen, H. Levy, R. Jeanloz, V. Schenker and G. Pincus: *Recent Progress Hormone Res.* **6:** 215. 1951.
22. Acevedo, H. F., O. Dominguez and L. T. Samuels: 41st Meeting of the Endocrine Society, Atlantic City, N. J. June 4–6, 1959.
23. Romanoff, E. B., P. Hudson and G. Pincus: *J. Clin. Endocrinol. and Metab.* **13:** 1546. 1953.
24. Bloch, E., R. I. Dorfman and G. Pincus: *Proceedings Soc. Exper. Biol. and Med.* **85:** 106. 1954.
25. Axelrod, L. R. and J. W. Goldzieher: *J. Clin. Endocrinol. and Metab.:* in press.
26. Deulofeu, V. and J. Farrari: *Z. physiol. Chem.* **226:** 192. 1934.
27. Haussler, E. P.: *Helv. chim. Acta* **17:** 531. 1934.

NOTES AND COMMENTS

EFFECT OF ADRENAL CORTICAL HORMONES ON ERUPTION RATE OF INCISOR TEETH IN THE RAT[1]

ABSTRACT

The influence of the adrenal glands on the eruption rate of adult rat maxillary incisor teeth was investigated. Administration of cortisone to normal controls in graded doses resulted in an enhanced eruption rate. Adrenalectomy was followed by a decreased eruption rate which was rapidly returned to normal by cortisone treatment, but not by desoxycorticosterone acetate. Hypophysectomy also caused a diminished eruption rate which was rapidly corrected by cortisone administration. These findings demonstrate the importance of the glucocorticoid type of adrenal steroids on incisor eruption in the adult rat.

Rat incisor teeth continue to erupt throughout life with the exposed portions being kept to a fairly constant length by attrition. The eruption rate is known to be diminished in the absence of the hypophysis or thyroid and restored toward normal by replacement therapy with growth hormone or thyroxine respectively (1, 2, 3). Parmer *et al.* reported premature incisor emergence into the oral cavity in newborn rats treated with cortisone (4).

Information being scant regarding the influence of the adrenal glands on the eruption rate, experiments were designed to establish whether such relationship exists.

MATERIALS AND METHODS

The extent of linear eruption of the maxillary incisor teeth was measured at weekly intervals in male albino rats of the Holtzman strain by the method of Schour and Van Dyke (1). Under light ether anesthesia, a fine mark was cut into the labial surface of the right maxillary incisor at the gingival margin with a Swiss jeweler's file at seven-day intervals. The distance between the last two marks was measured with a vernier caliper to the nearest 0.1/mm.

Effect of cortisone acetate in intact rats

Fifty rats weighing 270–300 gm. were randomized into five groups of 10 rats each and treated as follows: Group A—untreated; Groups B, C, D and E were treated respectively with 1, 2.5, 5 and 10 mg. cortisone acetate daily. The eruption rate was measured weekly for four consecutive weeks. At the end of the first, second and third weeks two animals selected at random from each group were killed, and the upper incisors and surrounding alveolar bone were carefully separated from the skull. One incisor was prepared for histochemical demonstration of alkaline phosphatase activity (5), and the other, for general histological study, with sections alternately stained with hematoxylin and eosin, and Heidenhains azan. The tooth structure, surrounding alveolar bone, connective tissue and vascular channels were studied.

Effect of cortisone acetate on hypophysectomized rats

The eruption rate was determined in 16 rats just prior to and for 26 days after hypophysectomy. Beginning at the 26th postoperative day, 2.5 mg. cortisone acetate were administered daily for two weeks to 9 of the 16 operated animals. The remaining 7 animals served as untreated, operated controls. Additional control data were provided by a group of 7 intact, untreated rats.

Effect of cortisone acetate and desoxycorticosterone acetate (DCA) administration on adrenalectomized rats

Forty-one rats, weighing 265–290 gm. were used; 31 were bilaterally adrenalectomized and 10 sham operated. All animals were offered both tap water and 1% saline *ad libitum*. The ex-

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[1] Preliminary report presented at the International Association for Dental Research, March, 1955.

tent of eruption was measured initially for one week prior to and one week following the operations. The adrenalectomized rats were then randomized into three groups and treated for 7 days as follows: 7 received 0.25 mg./day DCA; 8 received 2.5 mg./day cortisone acetate, and 16 were untreated. At the end of the second postoperative week a terminal measurement of eruption rate was made in all animals.

RESULTS

Cortisone acetate in intact rats—Cortisone acetate in all doses used caused the eruption rate to increase significantly ($P < 0.01$) over that observed in the untreated animals (Table 1). However, the group receiving 1 mg. did not differ significantly from that given 2.5 mg. and similarly the group receiving 5 mg. did not differ significantly from that receiving 10 mg. The 1 and 2.5 mg. groups were significantly different from the 5 and 10 mg. groups. This dose response was noted during the first two weeks of treatment.

TABLE 1. INCISOR ERUPTION RATE (E.R.) OF INTACT RATS TREATED WITH CORTISONE ACETATE

Dose (mg./day)	Week 1			Week 2			Week 3			Week 4		
	No. rats	Body wt. (gm.)	E.R. (mm.)	No. rats	Body wt. (gm.)	E.R. (mm.)	No. rats	Body wt. (gm.)	E.R. (mm.)	No. rats	Body wt. (gm.)	E.R. (mm.)
Control	10	303	2.5 ± .3*	8	336	2.9 ± .2	6	354	2.8 ± .2	4	383	2.7 ± .1
1.0	10	303	3.05 ± .3	8	311	3.3 ± .3	6	314	3.26 ± .1	4	328	3.0 ± .1
2.5	10	295	3.18 ± .2	8	290	3.5 ± .2	6	282	2.48 ± .3	4	271	3.12 ± .1
5.0	10	293	3.6 ± .3	8	276	3.7 ± .1	6	253	3.23 ± .2	4	243	2.8 ± .2
10.0	10	283	3.4 ± .3	7	246	3.6 ± .3	5	235	2.9 ± .2	3	223	2.06 ± .3

* Standard deviation.

A falling-off in eruption rate was noted in all animals during the third week, being more marked in those receiving the higher doses of the steroid. In the fourth week the eruption rate diminished even more markedly. A decrease in body weight relative to the amount of cortisone administered was observed in those rats receiving more than 1.0 mg. steroid daily. The group treated with 1 mg./day cortisone acetate continued to gain weight, though in smaller increments than the controls.

No differences in either morphology or alkaline phosphatase activity were observed between the sections of the cortisone treated and the control groups.

Cortisone acetate in hypophysectomized rats—The eruption rate at 26 days after hypophysectomy was 36% that of the intact control animals. The administration of 2.5 mg. cortisone acetate daily returned the eruption rate to approximately control values. In untreated hypophysectomized rats eruption continued to decline slowly during the 26th to 40th postoperative days (Fig. 1).

Adrenocortical steroids in adrenalectomized rats—Adrenalectomy reduced the eruption rate to 69% of that noted in the sham operated controls one week after operation. Administration of 0.25 mg. DCA daily for one week had no significant effect on eruption rate. Injection of 2.5 mg./day of cortisone acetate, however, for the same time interval returned the eruption rate of adrenalectomized animals to the control level (Table 2).

COMMENT

These data demonstrate the importance of the adrenal glucocorticoids in incisor eruption in the rat. Since both the adrenalectomized and the hypophysectomized animals lost weight, it is of interest that Bryer (6) noted that starvation *per se* did not affect eruption adversely. The changes in the alveolar bone and periodontal membrane in cortisone-treated rats noted by Goldsmith *et al.* (7, 8) were not observed in this study. It is therefore unlikely that the effect of cortisone on tooth eruption is secondary to its

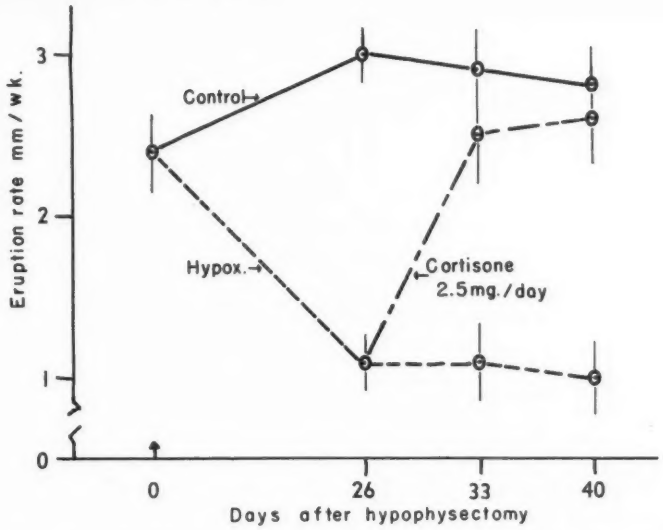

FIG. 1. The effect of cortisone acetate on incisor eruption rate of hypophysectomized adult rats. The steroid was administered from the 26th to the 40th day. The vertical lines through each point on the curves indicate the range observed in the measurements of eruption rate among animals in each group.

TABLE 2. INCISOR ERUPTION RATE (E.R.) IN ADRENALECTOMIZED RATS TREATED WITH CORTISONE AND DCA

Group	No. rats		Week 1	No. rats	Week 2
Controls	10	Body wt. (gm.)	284	10	290
		E.R. (mm.)	2.9 ± .2*		2.8 ± .2
Adrenx**	31	Body wt. (gm.)	263	16	256
		E.R. (mm.)	2.0 ± .2		1.9 ± .3
Adrenx + 0.25 mg./day DCA		Body wt. (gm.)		7	287
		E.R. (mm.)			2.1 ± .3
Adrenx + 2.5 mg./day cortisone acetate		Body wt. (gm.)		8	267
		E.R. (mm.)			3.1 ± .3

* Standard deviation.
** After Week 1, this group was redistributed as described in the text.

influence on the tooth's surrounding bone and connective tissue, but may be a direct one influencing the eruptive process in a manner not yet understood.

LEONARD GARREN[2] AND ROY O. GREEP

Biological Laboratories
Harvard School of Dental Medicine
Boston 15, Massachusetts

[2] Present address: National Institutes of Health, Bethesda, Md.

REFERENCES

1. Schour, I. and H. B. Van Dyke: *Am. J. Anat.* **50**: 397. 1932.
2. Baume, L. J., H. Becks and H. M. Evans: *J. Dent. Res.* **33**: 80. 1954.
3. Garren, L. and R. O. Greep: *Proc. Soc. Exper. Biol. & Med.* **90**: 652. 1955.
4. Parmer, L. G., F. Katonah and A. A. August: *Proc. Soc. Exper. Biol. & Med.* **77**: 215. 1951.
5. Greep, R. O., C. J. Fischer and A. Morse: *J. Am. Dent. Assoc.* **36**: 427. 1948.
6. Bryer, L. W.: *International Dent. J.* **7**: 432. 1957.
7. Goldsmith, E. D. and S. Stahl: *J. Dent. Res.* **32**: 699. 1953.
8. Goldsmith, E. D. and L. Ross: *Acta Endocrinol.* **22**: 23. 1956.

SYNTHESIS AND PHYSIOLOGICAL ACTIVITY OF SOME NEW ANALOGUES OF THYROXINE

ABSTRACT

The lactic acid analogues of 3,5-diiodothyronine, 3,5,3′-triiodothyronine and thyroxine have been synthesized and assayed by the goiter prevention method in rats. In this test, the T4 and T3 analogues are considerably less active than either the corresponding acetic or propionic acids.

Two new halogenated thyronines have been prepared: 3,3′-dichloro-5,5′-diiodo-L-thyronine and 3,3′-dibromo-5,5′-diiodo-L-thyronine; both have approximately equal potencies in the goiter prevention test of about ⅛ that L-thyroxine.

During the course of some work by Tata (1) on the effect of radiation upon I^{131} labeled thyroxine and 3,5,3′-triiodothyronine in solution, it became desirable to synthesize 3,5-diiodo-4-(4′-hydroxy-3′-iodophenoxy) phenyllactic acid (3,5,3′-triiodothyrolactic acid, T3La). The lactic acid analogue of thyroxine (T4La) had previously been synthesized by Saul and Trikojus (2).

It was also thought of interest to determine the physiological activities of thyronines containing mixed halogen atoms, in which each ring had two different halogen atoms. For these tests, 3,3′-dichloro-5,5′-diiodothyronine and 3,3′-dibromo-5,5′-diiodothyronine were prepared.

EXPERIMENTAL

(1) *Syntheses*[1]

3,5-Diiodothyropyruvic acid (*KT2*) was obtained by the method of Kennedy (3) by formation of the oxazolone from N-chloroacetyl-3,5-diiodothyronine followed by acid hydrolysis.

3,5-Diiodothyrolactic acid (*T2La*) was prepared by reduction of KT2 with potassium borohydride in methanol buffered with aqueous bicarbonate.

3,5,3′-Triiodothyrolactic acid (T2La) was iodinated in a mixture of methanol and ammonia as for the preparation of triiodothyroacetic acid (Triac) (4).

3,5,3′,5′-Tetraiodothyrolactic acid (*T4La*) was prepared from T2La by the method used for the synthesis of tetraiodothyroacetic acid (5) and by the method of Saul and Trikojus (1). Both products had the same m.p. (176–177°) and a mixed m.p. was not depressed.

3,3′-Dichloro-5,5′-diiodo-L-thyronine was prepared by aerobic incubation of *N-acetyl-3-chloro-5-iodo-L-tyrosine* in 0.2M borate buffer pH 7.5 at 37° for 3 months, followed by acid hydrolysis of the N-acetyl thyronine derivative (see 6). The product was crystallized as the hydrochloride.

3,3′-Dibromo-5,5′-diiodo-L-thyronine was prepared from *N-acetyl-3-bromo-5′-iodo-L-tyrosine* by the method described above. The compound was crystallized as the hydrochloride.

(2) *Physiological tests*

All compounds were tested in the goiter prevention assay in rats at two dose levels (except for $T2I_4$), using L- or DL-thyroxine (T4) as the standard compound. Estimations of potency were made by the parallel line method. The doses used were: DL-T4, 2.5 and 5 μg.; L-T4, 1.25 and 2.5 μg.; T2La, 100 μg.; T3La and T4La, 10 and 20 μg.; the mixed halogenated thyronines, 10 and 20 μg.

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[1] Synthetic methods for all new compounds will be described in detail elsewhere.

RESULTS

The potencies of the above compounds are shown in Table 1.

TABLE 1

Compound	Potency
DL-T4	1
T2La	<0.025
T3La	0.25
T4La	0.16
L-T4	1
3,3′-dichloro-5,5′ -diiodo-L-thyronine	0.164
3,3′-dibromo-5,5′ -diiodo-L-thyronine	0.178

COMMENT

The physiological activities of the lactic acid analogues of the thyroid hormones are similar to those of the corresponding keto acids (7) and, in the thyroxine series, lower than other analogues possessing 3-carbon side chains, when assayed by the goiter prevention method.

It has been pointed out by Bruice *et al.* (8) that the potency of thyroxine analogues in amphibia decreases with increasing acidity of the side chain. From previously reported data (9, 10) and from the results presented here, this does not appear to be generally true in the goiter prevention assay in rats (Table 2).

TABLE 2

Tetraiodo compound	Potency	Triiodo compound	Potency	pK (side chain)
DL-T4	1	DL-T3	5	—
Tetraiodo-acetic	1	Triiodo-acetic	1	4.77
Tetraiodo-butyric	0.14	Triiodo-butyric	0.14	4.83
Tetraiodo-propionic	0.28–0.5	Triiodo-propionic	0.56	4.87
Tetraiodo-acrylic	0.4 –0.5	—	—	4.26
Tetraiodo-lactic	0.16	Triiodo-lactic	0.25	3.87
Tetraiodo-pyruvic	0.13	Triiodo-pyruvic	0.53	2.50

It can be seen that T4 and T3 analogues with side chains of comparable acidities may have very different potencies (i.e. the acetic, propionic and butyric analogues); here, activity appears to be inversely related to the length of the side chain.

In the series of analogues with 3-carbon side chains, propionic, acrylic, lactic and pyruvic, the most acidic thyroxine derivatives, namely the lactic and keto acids, have only about $\frac{1}{2}$ to $\frac{1}{3}$ of the activity of the propionic and acrylic acids; however if one considers the corresponding acids derived from T3, this relationship no longer holds, since the most acidic compound, KT3, is as active as the least acidic, the propionic acid analogue.

With regard to the potencies of 3,3′-dichloro-5,5′-diiodothyronine and 3,3′-dibromo-5,5′-diiodothyronine, little emerges to help in the formulation of any rule that might lead to the prediction of biological potency. The similarity in their activities is perhaps somewhat surprising in view of the known decrease in activity when chlorine is substituted for bromine in the thyronine molecule (11). Further, it is not obvious why the potency of 3,3′-dibromo-5,5′-diiodothyronine should be $2\frac{1}{2}$ times that of 3:5-diiodo-3′-5′-dibromothyronine while the potency of 3,3′-dichloro-5,5′-diiodothyronine is only $\frac{1}{4}$ that of 3,5-diiodo-3′-5′-dichlorothyronine.

It would appear that in the mammal, the thyromimetic activity of compounds structurally related to the thyroid hormones is a complex function, dependent not only on the nature of the compound itself, but also on its availability to its target organ and perhaps to its rate of degradation which might lead to the rapid destruction of the active site in the molecule.

ROSALIND PITT-RIVERS

National Institute for Medical Research
Mill Hill, London, England

REFERENCES

1. TATA, J. R.: *Clin. Chim. Acta* **4**: 427. 1959.
2. SAUL, J. A. AND V. M. TRIKOJUS: *Biochem. J.* **42**: 80. 1948.
3. KENNEDY, T. H.: unpublished.
4. PITT-RIVERS, R.: *Lancet* **2**: 234. 1953.
5. HARINGTON, C. R. AND R. PITT-RIVERS: *Biochem. J.* **50**: 438. 1952.
6. PITT-RIVERS, R.: *Biochem. J.* **43**: 223. 1948.
7. PITT-RIVERS, R.: unpublished.
8. BRUICE, T. C., N. KHARASCH AND R. J. WINZLER: *Arch. Biochem.* **62**: 305. 1956.
9. SELENKOW, H. A. AND S. P. ASPER, JR.: *Physiol. Rev.* **35**: 426. 1955.
10. STASILLI, N. R., R. L. KROC AND R. I. MELTZER: *Endocrinology* **64**: 62. 1959.
11. MUSSETT, M. V. AND R. PITT-RIVERS: *Metabolism* **6**: 18. 1957.

THE EFFECTS OF ADRENAL CORTICOIDS UPON THE URINARY EXCRETION OF AMMONIA IN THE ADRENALECTOMIZED, ACIDOTIC RAT[1,2]

ABSTRACT

The twenty-four hour urinary excretion of ammonia was determined in a series of male, adrenalectomized rats following the production of acidosis with ammonium chloride, and after the administration of various adrenal cortical steroids. Ammonia excretion in the steroid-treated, acidotic rats was compared with ammonia excretion in acidotic, intact and adrenalectomized controls receiving no steroid. Concurrent urinary excretion of sodium and potassium were also measured.

All of the steroids used in this study caused increases in urinary ammonia excretion.

The adrenalectomized rat excretes less ammonia in the urine when subjected to acid loading than does the intact rat under otherwise identical laboratory conditions (1, 2). This deficiency in urinary ammonia excretion can be corrected by the administration of desoxycorticosterone acetate or whole adrenal cortex extract (2).

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[2] The work reported here was performed during the tenure of a Karr Fellowship, held by the senior author, which was provided by Smith, Kline and French Laboratories, and includes some of the data presented by the senior author in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Physiology at the University of Wisconsin.

The present study was undertaken in an attempt to determine the effects of other adrenal corticoids upon urinary excretion in the adrenalectomized, acidotic rat.

METHODS

Holtzman male rats weighing 325–350 grams were used. All but two groups of eight rats each were bilaterally adrenalectomized. All animals were maintained on Rockland rat diet and tap water *ad libitum*. Adrenalectomized rats received tap water containing 1% NaCl. Three days were allowed for recovery before treatment was started. Beginning on the fourth day, all rats in the groups receiving ammonium chloride were injected subcutaneously twice daily with a 10–12 hour interval between injections. Each dose consisted of 1.25 meq. NH_4Cl in 0.5 ml. H_2O, or a total of 2.5 meq. NH_4Cl per day. Rats in the steroid-treated groups were given a second injection subcutaneously, at a different body site, of steroid dissolved or suspended in corn oil. Steroids were also injected twice daily with half of the dosage given at each injection time. Each steroid injection was given in a volume of 0.25 ml.

On the third day of treatment the rats were injected with NH_4Cl and/or steroid and placed in individual metabolism cages. While in the metabolism cages, the rats received no food and were given distilled water for drinking. From ten to twelve hours thereafter, the rats were removed, given their second injections, and returned to their cages. Urine was collected for 24 hours under toluene. Three days of treatment, with urine collection during the third day, was considered to be a sufficient time interval for the stabilization of urinary patterns to occur in response to the given degree of acid loading. Following the collection period, the funnels of the cages and the cage bottoms were rinsed with distilled water and the rinsings were added to their respective urine specimens. Aliquots of each specimen were then analyzed for ammonia, sodium, and potassium content. Ammonia was determined colorimetrically by Nesslerization following its separation from urine by the Permutit resin method employing a modification (3) of the method originally reported by Folin and Bell (4). Sodium and potassium were determined by flame photometry.

Following the urine collection period, all adrenalectomized rats were autopsied in order to verify the completeness of adrenalectomy.

RESULTS AND DISCUSSION

As shown in Table 1, adrenalectomy alone did not cause any change in urinary ammonia excretion when compared with the excretion of ammonia in the normal intact rat. The increase in urinary sodium and the decrease in urinary potassium are consistent with the characteristic changes in excretion of these cations observed following adrenalectomy. Induction of acidosis (Table 2), however, resulted in a significant difference between urinary ammonia levels in intact animals and adrenalectomized animals. Table 2 lists the data obtained when urinary levels of ammonia, sodium, and potassium were determined in steroid-treated rats which had also been given ammonium chloride. All of the steroids which were employed caused increased urinary ammonia output when compared with adrenalectomized control animals. Several of the corticoids increased urinary ammonia to levels higher than that found in the intact control animals. Hydrocortisone was given at two different dosages and the results obtained suggest the possibility that a dose-response relationship may exist. The difference in urinary ammonia observed between the two hydrocortisone-treated groups is statistically significant at the 5% level by "t" test analysis (5). Aldosterone was effective as a stimulator of urinary ammonia output and would seem to be a very potent agent in this respect, since the response observed was obtained with a racemic mixture, and at a dosage which was only 1/80 by weight of that employed with most of the other steroids (1 mg./day).

No clear cut correlation between urinary levels of sodium and ammonia output was observed. High ammonia did not necessarily occur with low sodium and vice-versa. The data perhaps offer some suggestion that urinary ammonia and potassium vary in the same direction. High urinary potassium seems to occur, more often than not, when high urinary ammonia is present. This can be seen in the groups which received hydrocortisone (2 mg.), aldosterone, and cortisone. The rats which received hydrocortisone (1 mg.) and 9-α-fluorohydrocortisone have mean values for urinary potassium which

TABLE 1. EFFECT OF ADRENALECTOMY AND HYDROCORTISONE UPON URINARY EXCRETION OF AMMONIA, SODIUM AND POTASSIUM

meq./24 hrs. (mean ± standard deviation)

Group	No. of rats	NH_3-N	Na	K
Intact	8	0.58 ± 0.13	0.87 ± 0.20	1.55 ± 0.24
Adrenx	8	0.57 ± 0.12	2.77 ± 0.49***	1.03 ± 0.24***
Adrenx Hydrocortisone	7	0.81 ± 0.14**	2.35 ± 0.33**	1.65 ± 0.20***

** <0.01.
*** <0.001.

TABLE 2. EFFECTS OF ADRENAL CORTICOIDS UPON URINARY AMMONIA, SODIUM AND POTASSIUM IN RATS TREATED FOR THREE DAYS WITH AMMONIUM CHLORIDE (2.5 MEQ/DAY)

meq/24 hrs. (mean ± standard deviation)

Group	Steroid	Dose/ Day	No. of Rats	NH_3-N	In[1]	Ad[2]	Na	In	Ad	K	In	Ad
Intact	None	—	8	1.41 ± 0.22			0.91 ± 0.14			1.53 ± 0.16		
Adrenex	None	—	8	1.15 ± 0.17	*		1.77 ± 0.35	***		1.34 ± 0.18	*	
Adrenex	DOCA	1 mg.	8	1.57 ± 0.28	—	***	0.57 ± 0.22	**	***	1.01 ± 0.13	***	**
Adrenex	Hydrccortisone	1 mg.	8	1.91 ± 0.16	***	***	2.13 ± 0.43	***	—	1.52 ± 0.30	—	—
Adrenex	Hydrocortisone	2 mg.	8	2.30 ± 0.38	***	***	2.00 ± 0.35	***	—	1.82 ± 0.20	**	***
Adrenex	Corticosterone	1 mg.	8	1.54 ± 0.29	—	**	2.00 ± 0.34	***	—	1.45 ± 0.20	—	—
Adrenex	d, l aldosterone-21-monoacetate	12.5 μg.	8	1.79 ± 0.19	**	***	1.38 ± 0.59	**	*	1.63 ± 0.19	—	**
Adrenex	Cortisone	1 mg.	8	1.95 ± 0.28	***	***	2.38 ± 0.66	***	*	1.74 ± 0.18	*	***
Adrenex	9α Fluoro hydrocortisone	100 μg.	8	2.26 ± 0.26	***	***	1.41 ± 0.37	**	—	1.46 ± 0.32	—	—

[1] In—Significance when compared to Intact Controls.
[2] Ad—Significance when compared to Adrenex Controls.
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, — not significant.

are greater than the corresponding value for the adrenalectomized controls, but the differences are not statistically significant, possibly due to greater variation within these particular groups as evidenced by larger standard deviations.

Table 1 presents the results obtained when non-acidotic, adrenalectomized rats were given hydrocortisone for three days and urinary ammonia, sodium, and potassium were determined on the third day of treatment. These data show that hydrocortisone can increase urinary ammonia even in the absence of acidosis.

The results obtained in this study indicate that the deficiency in urinary ammonia output which occurs in the adrenalectomized rat when it is subjected to acid loading can apparently be corrected by adrenal corticoids which have either "glucocorticoid" or "mineralocorticoid" properties as their predominant biological activity. However since pharmacologic doses of steroids were used in order to accentuate effects, it is possible that the effects observed are not due to the predominant activity of the steroids, but to activity which is seen only when relatively large amounts of corticoid are given. Since aldosterone stimulated urinary ammonia output to a degree similar to that seen with the other steroids when it was given at a dosage which was 1/160 (active isomer weight basis) of that with other corticoids, it would seem that this steroid is probably more important than others of the adrenal cortex in the physiological maintenance of the renal ammonia mechanism.

Acknowledgment

We wish to express our gratitude to Miss Dorothy R. Hagedorn and Mrs. Una K. Loschky for their valuable technical assistance in carrying out the determinations of sodium and potassium which are reported in this study.

ALFRED BORIS[3] AND EDGAR S. GORDON

Departments of Physiology and Medicine
University of Wisconsin School of Medicine
Madison, Wisconsin

REFERENCES

1. SARTORIUS, O. W., D. CALHOON AND R. F. PITTS: *Endocrinology* **51:** 444. 1952.
2. SARTORIUS, O. W., D. CALHOON AND R. F. PITTS: *Endocrinology* **52:** 256. 1953.
3. HAWK, P. B., B. L. OSER AND W. H. SUMMERSON: Practical Physiological Chemistry, 12th edition, Blakiston Company, Philadelphia, 1947.
4. FOLIN, O. AND R. D. BELL: *J. Biol. Chem.* **29:** 329. 1917.
5. SNEDECOR, G. W.: Statistical Methods, 5th edition, Iowa State College Press, Ames, 1956.

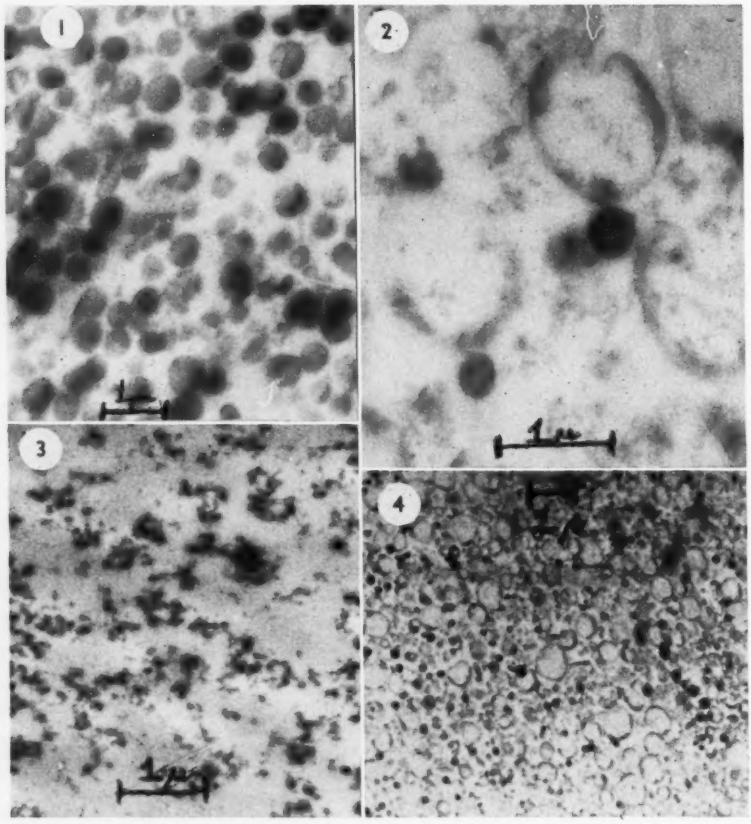
[3] Present Address: Department of Pharmacology, Hoffmann-La Roche, Inc., Nutley, New Jersey.

CORRECTION

In the article by A. C. J. Bergers and Choh Hao Li, entitled "Amphibian Ovulation *in vitro* Induced by Mammalian Pituitary Hormones and Progesterone" which appeared in the February 1960 issue of ENDOCRINOLOGY, the name of the senior author should read A. C. J. Burgers.

ERRATUM

J. H. U. Brown *et al.* in the article "Studies on the Intracellular Localization of Anterior Pituitary Hormones," in ENDOCRINOLOGY **66:** 1, 1960 inadvertently included electronmicrographs which were actually in part previously published pictures of beef pituitary (*Amer. J. Physiol.* **188:** 25, 1957). The authors wish to apologize for their obvious error and provide correct micrographs of pig pituitary glands.



FIGS. 1–4. Electronmicrographs of pig pituitary prepared by Dr. Duane Belt, Department of Anatomy. Fig. 1. Section of a pellet of secretory granules. Fig. 2. Mitochondria from pig pituitary. Fig. 3. Section taken from a pellet of the microsomal fraction. Fig. 4. Low power view of mitochondrial pellet showing the degree of contamination with other fractions.

THE ENDOCRINE SOCIETY

1960 Annual Meeting

The Forty-Second Annual Meeting of The Endocrine Society will be held in the Eden Roc Hotel, Miami Beach, Florida, Thursday, Friday, and Saturday, June 9, 10, 11, 1960.

The Chairman of Local Arrangements is Dr. Kenneth Savard.

Scientific Sessions will be held from 9:00 A.M. to 5:00 P.M. daily and in addition there will be simultaneous afternoon sessions. The annual dinner is scheduled for Friday, June 10 at 7:30 P.M. preceded by cocktails at 6:30 P.M.

All members are urged to make their hotel reservations immediately. The Eden Roc will hold 250 bedrooms and the adjoining hotels, the Montmartre Hotel and Lucerne Hotel will hold 75 rooms each, for members until May 1, 1960, after which time the hotel will not guarantee further reservations. Therefore it is imperative that you make your reservations now directly with the hotel, advising them of time and date of arrival and departure. If you plan on remaining through June 12th for the American Medical Association and other meetings, it is imperative that your reservation be made from June 12th on, directly through the A.M.A. Housing Bureau, c/o Miami Beach Convention Bureau, 1700 Washington Avenue, Miami Beach, Florida, since no reservation beyond this date can be accepted directly by the hotels. Make your reservations now and avoid disappointment. Hotel reservation card will be mailed to members shortly after January 1, 1960.

Final program, membership card and advance registration forms will be sent on May 1st 1960 to members whose current dues have been paid.

1960 AWARDS

The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by the Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors.

Fred Conrad Koch Award

During the past year a substantial legacy has been bequeathed the Society by the late Elizabeth Koch for the purpose of establishing the Fred Conrad Koch Memorial Fund in memory of her late husband, distinguished service professor of physiological chemistry at the University of Chicago and pioneer in the isolation of the androgens. This is to be the highest honor of the Endocrine Society and is to be represented by a medal that is to be known as the Koch Medal of The Endocrine Society. The medal and honorarium of $3,500 is to be given annually to an individual for work of special distinction in endocrinology.

The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada. This award will replace the Medal of the Endocrine Society which was established in 1954 and presented to Dr. Carl Moore in 1955, Dr. Frederick L. Hisaw in 1956, Dr. Joseph C. Aub in 1957, Dr. I.L. Chaikoff in 1958 and Dr. Wilbur W. Swingle in 1959. The Endocrine Medal replaced the E. R. Squibb Award which was formerly the highest honor bestowed by the Society. Past recipients of the Squibb Award were Dr. George W. Corner in 1940, Dr. Philip E. Smith in 1941, Dr. Fred C. Koch, 1942, no award was given in 1943, Dr. E. A. Doisy in 1944, Dr. E. C. Kendall in 1945, Dr. Carl O. Hartman in 1946, Drs. Carl F. and Gerty T. Cori in 1947, Dr. Fuller Albright in 1948, Dr. Herbert Evans in 1949, Dr. C. N. H. Long in 1950, Dr. J. B. Collip in 1951, Dr. James H. Means in 1952 and Dr. David Marine in 1953.

The Ciba Award

The Ciba Award, to recognize the meritorious accomplishments of an investigator who has not attained his 36th birthday by June 1st of the year the award is presented, in the field of clinical or preclinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood, 1945 Dr. Jane A. Russell, 1946 Dr. Martin M. Hoffman, 1947 Dr. Choh Hao Li, 1948 Dr. Carl Heller, 1949 Dr. George Sayers, 1950 Dr. Oscar M. Hechter, 1951 Dr. Albert Segaloff, 1952 Dr. Seymour Lieberman, 1953 Dr. Sidney Roberts and Dr. Clara Szego (Mrs. Roberts), 1954 Dr. Isadore M. Rosenberg, 1955 Dr. Jack Gross, 1956 Dr. Alfred M. Bongiovanni, 1957 Dr. Nicholas S. Halmi, 1958 Dr. Monte Arnold Greer, and 1959 Dr. Gordon L. Farrell. Prior to 1952 the Award was $1,200. It has now been increased to $2,500.

The Ayerst and the Squibb Fellowships

The Ayerst Fellowship was established in 1947 and the Squibb Fellowship in 1956. They are designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology.

Each Fellowship is awarded on alternate years and the stipend, which will not exceed $5,000 may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree, or candidates for either of these degrees, are eligible for appointment.

Applicants must submit the following information:

1. Evidence of scientific ability as attested by studies completed or in progress.
2. Recommendations from individuals familiar with the candidate and his work.
3. A proposed program of study.
4. Acceptance of the individual by the head of the department in which the Fellowship will be held.
5. A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

The Schering and the Upjohn Scholars of The Endocrine Society

The Council of The Endocrine Society has established a category of Scholars. These grants have been made available through the generosity of the Schering Corporation and the Upjohn Company, and will be awarded to established investigators and teachers in the field of endocrinology who wish to extend their opportunities for work either in this country or abroad.

The awards will not exceed $2,500 annually for each individual and will be granted on the basis of proposals submitted by the applicant. Such applications should include estimated financial needs. The funds may be used for travel, maintenance and other expenses.

Nominations

Nominations for the Fred Conrad Koch Award; the Ciba Award; and the Ayerst and the Squibb Fellowships may be made by any member of The Endocrine Society. They should be submitted on forms which may be obtained from the Office of the Secretary, 1200 North Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than October 15, 1959.

Proposals for appointments as Scholars of The Endocrine Society should be made in writing by the individual, and addressed to the secretary of the Society. They should be submitted by October 15, 1959.

The Awards Committee will meet in October and notice of awards to successful nominees and applicants will be made not later than December 1st.

ENDOCRINE SOCIETY PROGRAM*

JUNE 9–11, 1960

THURSDAY, JUNE 9, 1960

8:00 A.M.—Registration—Lobby—Eden Roc Hotel

I. 9:00 A.M.—ADRENAL

Cafe Pompeii

Chairman: *Dwight J. Ingle*

1. On the Mechanism by which Potassium Increases Secretion of Aldosterone.
   Donald S. Gann (by invitation), *Juan F. Cruz* (by invitation), *Alfred G. T. Casper* (by invitation) and *Frederic C. Bartter.*
2. Increased Aldosterone Secretion Following Decapitation and Subsequent Bleeding of Hypophysectomized Dogs.
   James O. Davis, Charles C. J. Carpenter (by invitation), *Carlos R. Ayers* (by invitation) and *Robert C. Bahn.*
3. Aldosterone Secretion Measured During Adrenal Vein Canulation in Humans.
   Walter H. Moran, Jr. and *Bernard Zimmermann.*
4. Stimulation by Aldosterone of Active Sodium Transport Across a Living Membrane.
   Jean Crabbé.
5. The Inotropic Action of d,l-Aldosterone on Isolated Mammalian Cardiac Preparations.
   Ralph D. Tanz (Introduced by George Sayers).
6. Effects of Mineralocorticoids on Na^{22} Uptake by Bone.
   David H. P. Streeten, David R. Rovner (by invitation), *Carlos Stevenson* (by invitation) and *Jerome W. Conn.*

INTERMISSION

7. Effects of Chronic Hepatic Venous Congestion on the Disappearance of Tritiated Aldosterone from Plasma.
   Carlos R. Ayers (by invitation), *James O. Davis, Ferol Lieberman* (by invitation) and *Charles C. J. Carpenter* (by invitation).
8. 21-Deoxysteroid Metabolites in Adrenocortical Dysfunction.
   R. I. Cox (Introduced by T. F. Gallagher).
9. Studies of Adrenocortical Function of Hirsute Women.
   Arnold M. Moses, Edward L. Klaiber (by invitation), *Julia Lobotsky* (by invitation), *Ross D. Jacobs* and *Charles W. Lloyd.*
10. Studies of Adrenal Nucleic Acids.
    E. D. Bransome, Jr. (by invitation) and *W. J. Reddy.*
11. Some Mechanisms of Steroid Hormone Action.
    Gordon M. Tomkins (Invited paper, 20 minutes).

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* Program Committee: E. B. Astwood, John L. Bakke, William H. Daughaday, T. S. Danowski, Peter H. Forsham, Gilbert S. Gordan, Roy Hertz, Sidney H. Ingbar, Dwight J. Ingle, Charles W. Lloyd, Warren C. Nelson, C. Alvin Paulsen, George Sayers, and Robert H. Williams, *Chairman.*

II. 2:00 P.M.—ADRENAL

Cafe Pompeii

Chairman: *Peter H. Forsham*

12. The Effects of Intracarotid Infusions of Cortisol on Impaired Water Diuresis of the Adrenalectomized Dog.
Charles R. Kleeman, Ralph E. Cutler (by invitation) and *Morton H. Maxwell* (by invitation).
13. Maturation of Adrenal Cortical and Medullary Function in the Dog.
B. T. Jackson (by invitation), *J. C. Melby, D. M. Hume* and *R. H. Egdahl.*
14. The Significance of the Concentration of Nonprotein-Bound Plasma Cortisol Concentration in Normal Subjects, Cushing's Syndrome, Pregnancy, and During Estrogen Therapy.
Richard P. Doe, Horace H. Zinneman (by invitation) and *Edmund B. Flink.*
15. Adrenal Secretion of 17 a-Hydroxyprogesterone in Normal Dogs.
Balwant D. Agashe (by invitation), *Robert G. Weaver* (by invitation) and *Kristen B. Eik-Nes.*
16. Steroid Studies During and After Prolonged Therapy with Zn-ACTH.
Eleanor Z. Wallace, Linda Leilop (by invitation), *Anne C. Carter* and *Harold A. Lyons* (by invitation).
17. Structure-Function Relationships of Steroids: Dissociation of Classical "Glucocorticoid" Properties by Structural Modification at Carbon-21.
John W. Kendall, Jr. (by invitation) and *Grant W. Liddle.*

INTERMISSION

18. Corticosteroids and Accumulation of Substrate by Isolated Rat Diaphragm: Possible Mechanism of Anti-Inflammatory Action.
Ira G. Wool.
19. The *in vitro* Reduction of 11 B-Hydroxy and 11-Ketosteroids by Liver Δ^4-Hydrogenases.
Enrico Forchielli (by invitation), *Keith Brown-Grant* (by invitation) and *Ralph I. Dorfman.*
20. Experimental Immunologic Adrenal Injury.
Jan Steiner (by invitation), *Douglas L. Schatz* (by invitation), *Bernard Langer* (by invitation) and *Robert Volpé.*
21. Effect of Hypercapnea on Plasma Epinephrine and Norepinephrine Concentration.
J. C. Rosenberg (by invitation), *B. Goott* (by invitation), *R. C. Lillehei* (by invitation), *F. Miller* (by invitation) and *Bernard Zimmermann.*
22. Inhibition of Pituitary ACTH Secretion as the Major Glucocorticoid Effect of a Synthetic Steroid.
Donald A. Holub, Fred H. Katz (by invitation) and *Joseph W. Jailer.*
23. Hormonal Influence on RNA Synthesis in the Liver.
K. E. Paschkis, A. Cantarow and *T. H. Williams* (by invitation).

III. 2:00 P.M.—PITUITARY

Mona Lisa Room

Chairman: *George Sayers and William H. Daughaday*

24. Further Purification Studies on Corticotropin Releasing Factor (CRF).
Andrew V. Schally, R. N. Andersen (by invitation), *Harry S. Lipscomb, John M. Long* (by invitation) and *Roger Guillemin.*

25. Apparent Dissociation Between Adrenal and Extra-Adrenal Actions of Corticotropin Chemically Altered by Periodate-Borohydride Treatment.
H. Angus Boright (by invitation), *Frank L. Engel, Jack L. Kostyo* and *J. Earle White, Jr.*
26. Modification of Pituitary-Adrenal Response to SU-4885 by Psychotropic Drugs.
Ernest M. Gold (by invitation), *John R. Kent* (by invitation) and *Peter H. Forsham.*
27. Studies on Adrenal Cortical and Anterior Pituitary Responsiveness.
T. Albert Farmer, Jr. (by invitation), *J. W. Herod, Jr.*, (by invitation), *James A. Pittman, Jr.* and *S. Richardson Hill, Jr.*
28. The Estimation of ACTH in Human Plasma.
V. K. Vance (by invitation), *W. J. Reddy, D. H. Nelson* and *G. W. Thorn.*
29. Disturbances of the Servo-Mechanism Regulating Adrenal Steroidogenesis in Patients with Cerebral Disease.
Jack H. Oppenheimer (by invitation), *Leonard V. Fisher* (by invitation) and *Joseph W. Jailer.*

INTERMISSION

30. Estimates of the Secretion Rate of Thyrotropin in the Normal and Hypothyroid Rat and Man.
J. L. Bakke and *N. Lawrence* (by invitation).
31. Extrathyroidal Actions of Pituitary Thyrotropin *in vitro:* Effects upon Rat Adipose Tissue.
Norbert Freinkel
32. Effect of Dietary Protein Deprivation on Pituitary Tropic Hormones in Normal and Acromegalic Subjects.
R. Palmer Howard and *Robert H. Furman.*
33. Neuroendocrinological Syndrome of Galactorrhea, Papilledema and Increased Intracranial Pressure.
Joseph F. Dingman, Eduardo Gaitan (by invitation), *Akira Arimura, Max C. Staub* (by invitation) and *Ruth Paterson* (by invitation).
34. Glass Paper Chromatographic Method for Vasopressin and Oxytocin in Human Plasma.
Akira Arimura and *Joseph F. Dingman.*
35. An Antidiuretic Effect of Chlorothiazide in Patients with Diabetes Insipidus.
Richard M. Portwood, Walter C. Lusk, E. C. Buckley (Introduced by *Frank L. Engel*).

IV. 7:30—TESTIMONIAL DINNER FOR ROY HOSKINS

Imperial Room

FRIDAY, JUNE 10, 1960

8:30 A.M.—Registration—Lobby

V. 9:00 A.M.—REPRODUCTION. CALCIUM METABOLISM.

Cafe Pompeii

Chairmen: *Roy Hertz and Gilbert S. Gordan*

36. Ultrastructure of the Placenta.
Edward W. Dempsey (Invited paper, 20 minutes).
37. 6-B-Hydroxy-Cortisol: Abnormally High Levels in Human Urine in Pregnancy and Toxemia.
Fred H. Katz (by invitation), *Andrew G. Frantz* (by invitation) and *Joseph W. Jailer.*

38. Progesterone Levels in the Rabbit Ovarian Vein Blood Throughout Pregnancy.
Girgis Mikhail (by invitation), *Matthew W. Noall* (by invitation) and *Willard M. Allen.*
39. The Nature of Uterine Phenol-Activated DPNH Oxidase.
Vincent P. Hollander and *Jean R. Beard* (by invitation).
40. Inhibition of Glucuronosyl Transferase by Steroid Hormones.
Robert M. Dowben and *David Y. Y. Hsia* (by invitation).

INTERMISSION

41. The Use of Norethynodrel in Fertility Control.
Edward T. Tyler.
42. New Criteria of "Target" Tissues for Steroid Estrogens.
Elwood V. Jensen.
43. Inhibitory Effects of Hypercalcemia on Secretion of Parathyroid Hormone.
Laurence H. Kyle, John J. Canary, Daniel H. Mintz (by invitation) and *Antonio C. de Leon* (by invitation).
44. Hypercalciuria Associated with "Steroid Withdrawal" in Cushing's Syndrome.
Samuel H. Bassett and *Stewart G. Tuttle* (by invitation).
45. Unifying Concepts of Parathyroid Hormone Action
A. Raymond Terepka, Charles Dowse and *William F. Neuman* (Invited paper, 20 minutes)

1:30 P.M.—ANNUAL BUSINESS MEETING

Cafe Pompeii

VI. 2:30 P.M.—PITUITARY. TESTES.

Cafe Pompeii

Chairmen: *Charles W. Lloyd and Warren O. Nelson*

46. Effects of Human Growth Hormone and Its Subfractions on Sodium Retention.
Edward G. Biglieri (by invitation), *Charles O. Watlington* (by invitation) and *Vincent C. di Raimondo.*
47. Hormonal Activity of Choriocarcinoma in Women and in Heterologous Transplants in Hamsters and Rats.
Roy Hertz, M. B. Lipsett and *S. J. Jackson* (by invitation).
48. Immunological Analysis of Ovine Pituitary LH.
Sheldon J. Segal.
49. The Action of Gonadotropic Hormone Preparation from Various Species in the Hypophysectomized Male Rhesus Monkey.
E. Knobil and *J. B. Josimovich* (by invitation).
50. The Maintenance of Luteotrophin Secretion by Means of Progesterone.
I. Rothchild.
51. Urinary Excretion of Luteinizing Hormone (LH) by Anxious Patients and Normal Subjects.
Harold Persky, Howard C. Jackson (by invitation), *Robert W. McCurdy* (by invitation) and *John Paul Brady* (by invitation).

INTERMISSION

52. Observations on the Endogenous Respiration of the Testicular Tissue.
E. Steinberger and *C. Wagner* (by invitation).

53. The Distribution of Testosterone-4-C^{14} in the Rat.
F. Parra (by invitation), *W. J. Reddy* and *G. W. Thorn.*
54. Effects of Androgens on Intermediary Metabolism in Muscle.
C. A. Villee, J. M. Loring (by invitation) and *J. M. Spencer* (by invitation).
55. *In vivo* Glycine-1-C^{14} Incorporation Studies of Mouse Kidney B-Glucuronidase in Response to Androgenic Stimulation.
Olive S. Pettengill (by invitation) and *William H. Fishman.*
56. Stimulation of Erythropoiesis by Androgenic Hormones.
B. J. Kennedy.
57. The Testis in Myotonic Dystrophy (Steinert's Disease).
Nicholas P. Christy, William D. Drucker (by invitation) and *William A. Blanc.*

VII. 2:30 P.M.—OVARY. THYROID.

Mona Lisa Room

Chairmen: *E. B. Astwood and S. H. Ingbar*

58. Comparative Hydrolysis of Estrogen Conjugates by Three Different Enzyme Preparations.
Avery A. Sandberg and *W. R. Slaunwhite, Jr.*
59. A Study of Estrogen Antagonism by MER-25.
Arthur Cutler (by invitation), *Jeanne A. Epstein* and *Herbert S. Kupperman.*
60. Discrepancies Between Various I^{131} Preparations in Measurement of Thyroid I^{131} Uptakes.
Alvin Taurog.
61. The *in vitro* Stimulation of Thyroidal Activity by Propylthiouracil.
Philip F. Mulvey, Jr. (by invitation), *D. Ward Slingerland* (by invitation) (Introduced by Belton A. Burrows).
62. The Thyroidal Organification of Radioiodine in the Presence of Inhibitory Amounts of Antithyroid Substance.
Marvin L. Mitchell, Jose A. Sanchez-Martin (by invitation), *Anne B. Harden* (by invitation) and *Mary E. O'Rourke* (by invitation).
63. Effect of Thyrotropin upon Thyroidal Iodide Release in Man and Rat.
I. N. Rosenberg, C. S. Ahn (by invitation) and *M. H. Chaflen* (by invitation).

INTERMISSION

64. Effects of Serotonin and Related Compounds on the Metabolism of Thyroid Hormones and Iodine.
Valerie Anne Galton (by invitation) and *Sidney H. Ingbar.*
65. The Role of Glucose and Insulin in Peripheral Thyroxine Degradation and Thyroidal Iodide Accumulation.
Herbert G. Rose (Introduced by John R. Hogness).
66. The Interaction of Serum Thyroxine During Pathologic Pregnancies with Ion Exchange Resins.
R. L. Nicoloff (by invitation), *J. T. Nicoloff* (by invitation) and *J. T. Dowling.*
67. Inheritance of Defective Organic Binding in Familial Goitrous Cretinism.
Rose H. Parker (by invitation) and *William H. Beierwaltes.*
68. Effect of Thyroid Deficiency on Amino Acid Transport in the Adenohypophysis.
Paulo A. Ayroza-Galvao (by invitation), *David M. Kipnis* and *William H. Daughaday.*
69. Dissociation of Calorigenic and Cholesterol Lowering Effects by Diiodothyroacetic Acid.

John R. Kent (by invitation), *Harry F. Hurd* (by invitation), *Edwin S. Stenberg* (by invitation) and *Peter H. Forsham.*

VIII. ANNUAL DINNER—Eden Roc Hotel

Dwight J. Ingle, President presiding

6:30 P.M.—Cocktails

Pavilion Room
Sponsored by the donors of the Awards of the Society:
Ciba, Ayerst, Upjohn and Squibb
Attendance limited to holders of dinner tickets

7:30 P.M.—Dinner—Imperial Room

Presentation of the 1960 Awards of the Society by Roland K. Meyer:
The Ciba Award
The Fred Conrad Koch Award
Squibb Fellowship
Upjohn Scholar
President's address: Dr Ingle

SATURDAY, JUNE 11, 1960

8:30 A.M.—Registration—Lobby

IX. 9:00 A.M.—DIABETES AND RELATED METABOLISM

Cafe Pompeii

Chairmen: *Thaddeus S. Danowski and Robert H. Williams*

70. The Diagnostic Value and Modus Operandi of Sodium Tolbutamide in Hypoglycemic States.
Stefan S. Fajans, Jack M. Schneider (by invitation), *David E. Schteingart* (by invitation) and *Jerome W. Conn.*
71. Transhepatic Balance of Insulin-Like Activity Following Hyperglycemia.
R. H. Egdahl and *H. L. Goldberg* (by invitation).
72. Studies on the Binding of Insulin to Adipose Tissue *in vitro.*
Rene Humbel (by invitation) and *Albert E. Renold.*
73. Effect of Plasma Protein Fractions from Normal and Hypophysectomized Subjects on Glucose Uptake by Muscle.
K. W. Taylor (Introduced by *A. E. Renold*).
74. Interference by Epinephrine, ACTH and Growth Hormone in Measurements of Insulin-Like Activity (ILA).
Jurgen Steinke (by invitation), *Gaston Zahnd* (by invitation) and *Albert E. Renold*
75. Hormonal Effects on Pathways of Glucose Metabolism of Endocrine Tissues *in Vitro.*
Ira Pastan, James B. Field and *Arnold N. Weinberg.*

INTERMISSION

76. Effects of Human, Monkey, Pig, and Sheep Growth Hormones on Glucose Metabolism in Adipose Tissue.
Albert I. Winegrad and *Walter N. Shaw* (Introduction by Francis D. W. Lukens).
77. The Lipolytic Response to Corticotropin.
C. H. Hollenberg (by invitation), *M. S. Raben* and *E. B. Astwood.*
78. An *in vitro* Effect of Cortisol on ACTH or Epinephrine Stimulated Adipose Tissue.

Bernard Leboeuf (by invitation) and *George F. Cahill, Jr.*

79. Influence of Hypophysectomy and Certain Hormones on a-Aminoisobutyrate (AIB) Transport in Adrenal and Diaphragm.
J. Eichhorn (by invitation), *I. D. K. Halkerston* (by invitation), *M. Feinstein* (by invitation) and *O. Hechter.*

80. Experience with Melatonin, a Pigment Cell Hormone from the Pineal.
James D. Case (by invitation), *Aaron B. Lerner* (by invitation), *M. Ruth Wright* (by invitation) and *Joseph S. McGuire* (by invitation). (Invited paper, 20 minutes)

X. 2:00 P.M.—CLINICAL SESSIONS

Cafe Pompeii

Chairman: *John E. Howard*

1. Control of Ovulation
Ovulation.
Charles W. Lloyd, Robert B. Greenblatt and *Gregory Pincus.*
2. Abnormalities of Sex Differentiation.
A. Classification and Etiology.
Melvin M. Grumbach.
B. Diagnosis and Selection of Gender of Rearing.
Lawson Wilkins.
C. Psychologic Aspects of Sex.
John Money.
D. Questions and Presentation of Cases.
3. Tests of Adrenocortical Function.
J. W. Jailer, L. J. Soffer, N. P. Christy and *R. E. Peterson.*

ANNOUNCEMENTS

6TH INTERNATIONAL CONGRESS OF INTERNAL MEDICINE

6th International Congress of Internal Medicine, August 24–27, 1960, Basle (Switzerland); organized by the International Society of Internal Medicine (President: Sir Russell Brain, F.A.C.P. Hon., London). President of the Congress: Prof. Dr. A. Gigon, Basle. Secretary: Prof. Dr. H. Ludwig, Basle.

Principal subjects of the Congress: Pathogenesis and Therapy in Edema—Enzymic Regulations in the Clinic. Panels: Interstitial Nephritis—Nephrosis—Diuretics—Diagnostic Value of Enzymes—Therapeutic Indications of the Enzymes—Serology in Rheumatic Diseases—Instruction in Internal Medicine. Lectures on subjects of free choice. Seventy leading internists from all over the world will take part in the main scientific programme by reading their papers or joining in the panel discussions.

Official languages: German, French, English. Simultaneous translation for the principal lectures. Programmes and registration forms for the congress may be obtained from the secretary of the 6th International Congress of Internal Medicine, Steinentorstrasse 13, Basle 10 (Switzerland). Registrations should reach the secretary if possible by April 30, 1960.

SOUTHWEST RESEARCH INSTITUTE

Unclassified symposium on the psychological and physiological aspects of space flight, May 26–27, 1960.

Arranged by Southwest Research Institute, sponsored by School of Aviation Medicine, USAF Aerospace Medical Center (ATC). Headquarters, Hilton Hotel, San Antonio, Texas.

EXCERPTA MEDICA FOUNDATION

NEW YORK OFFICE: THE NEW YORK ACADEMY OF MEDICINE BUILDING
2 EAST 103 STREET, NEW YORK 29, N. Y.

Our chief editors have decided to begin publication of a recorded medical journal to be published under the title "Voice of Medicine." We shall publish recordings of discussions and interviews taken during national and international medical congresses, clinical-pathological conferences, world health assemblies, medical meetings and symposia. We shall include discussions by leading specialists on subjects of current medical interest arranged by Excerpta Medica on an international level.

NEW BOOK REGISTER

CARCINOGENESIS, MECHANISMS OF ACTION. Ciba Foundation Symposium. Boston: Little, Brown and Company.

REGULATION OF CELL METABOLISM. Ciba Foundation Symposium. Boston: Little, Brown and Company.

IMPLANTATION OF OVA. Edited by P. Eckstein. Memoirs of the Society of Endocrinology. England: Cambridge University Press.

COMPARATIVE ENDOCRINOLOGY. Gorbman. Columbia-Cold Spring Harbor Symposium. Springfield: C. C Thomas Publishing Co.

DIE STERILITAT DER FRAU. Bickenbach and Doring. Stuttgart: Georg Thieme Verlag.

HORMONTHERAPIE IN DER FRAUENHEILKUNDE. Joachim Ufer. Berlin: Walter de Gruyter, & Co.

RECENT PROGRESS IN HORMONE RESEARCH. Proceedings of the Laurentian Hormone Conference 1958. Edited by Gregory Pincus. New York and London: Academic Press.

DE ENDEMISCHE KROP IN NEDERLAND. Koninklijke Van Gorcum & Company.

MAJOR ENDOCRINE DISORDERS. S. Leonard Simpson. New York: Oxford University Press.

PSYCHIATRY IN THE MEDICAL SPECIALTIES. Flanders Dunbar. New York: McGraw-Hill Book Company, Inc.

RECENT PROGRESS IN OXYTOCIN RESEARCH. B. Berde. Springfield: Charles C Thomas, Publishers.

METABOLIC HOMEOSTASIS. Nathan B. Talbot, Robert H. Richie and John D. Crawford. Cambridge: Harvard University Press.

CLINICAL DISORDERS OF HYDRATION AND ACID-BASE EQUILIBRIUM. Louis G. Welt. New York: The Ronald Press Co.

REPRODUCTION IN DOMESTIC ANIMALS. Edited by H. H. Cole and P. T. Cupps. New York and London: Academic Press.

GYNAECOMASTIA. Peter F. Hall. Australasian Medical Publishing Co., Lt. Sydney.

THE DIABETIC'S HANDBOOK. (Second Edition) Anthony M. Sindoni, Jr. New York: The Ronald Press.

MICROBIOLOGICAL REACTIONS. Ciba Foundation Study Group No. 2. Boston: Little, Brown and Co.

CANCER CURRENT LITERATURE INDEX. American Cancer Society, Inc. New York.

PROBLEMS ACTUELS D'ENDOCRINOLOGIE ET DE NUTRITION. Edited by H. P. Klotz and J. Tremolieres. Paris: Expansion Scientifique Francaise.

GUSTA ENDEMICA DISTROVIA ENDEMICA TIREOPATA SUB REDACTIA ACAD. St.-M. Milcu. Volume I. Editura Academiei Republicii Populare Romine.

GUSTA ENDEMICA DISTROFIA ENDEMICA TIREOPATA SUB REDACTIA ACAD. St.-M. Milcu. Volume II Editura Academiei Republicii Populare Romine.

OPERE ALESE. Vol. II. Endocrinologie Generala, Glanda Tiroida, Glandele Paratiroide, Si Timus. C. I. Parhon. Editura Academiei Republicii Populare Romine.

ENDOCRINOLOGIA GLANDELOR SALIVARE. Acad. C. I. Parhon and A. Babes i i. Petrea. Editura Academiei Republicii Populare Romine.

EPIFIZA Glanda Endocrina de Academician St.-M. Milcu. Editura Academiei Republicii Populare Romine.